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Saboulard et al.

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(45) **Date of Patent: Jan. 7, 2014**

(54) **VIII FACTORS FOR THE TREATMENT OF TYPE A HEMOPHILIA**

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PCT Pub. Date: **Oct. 30, 2008**

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(30) **Foreign Application Priority Data**

Feb. 23, 2007 (FR) 07 53450

(51) **Int. Cl.**

A61K 38/37 (2006.01)
C12Q 1/56 (2006.01)

(52) **U.S. Cl.**

USPC **514/14.1**; 435/13

(58) **Field of Classification Search**

None

See application file for complete search history.

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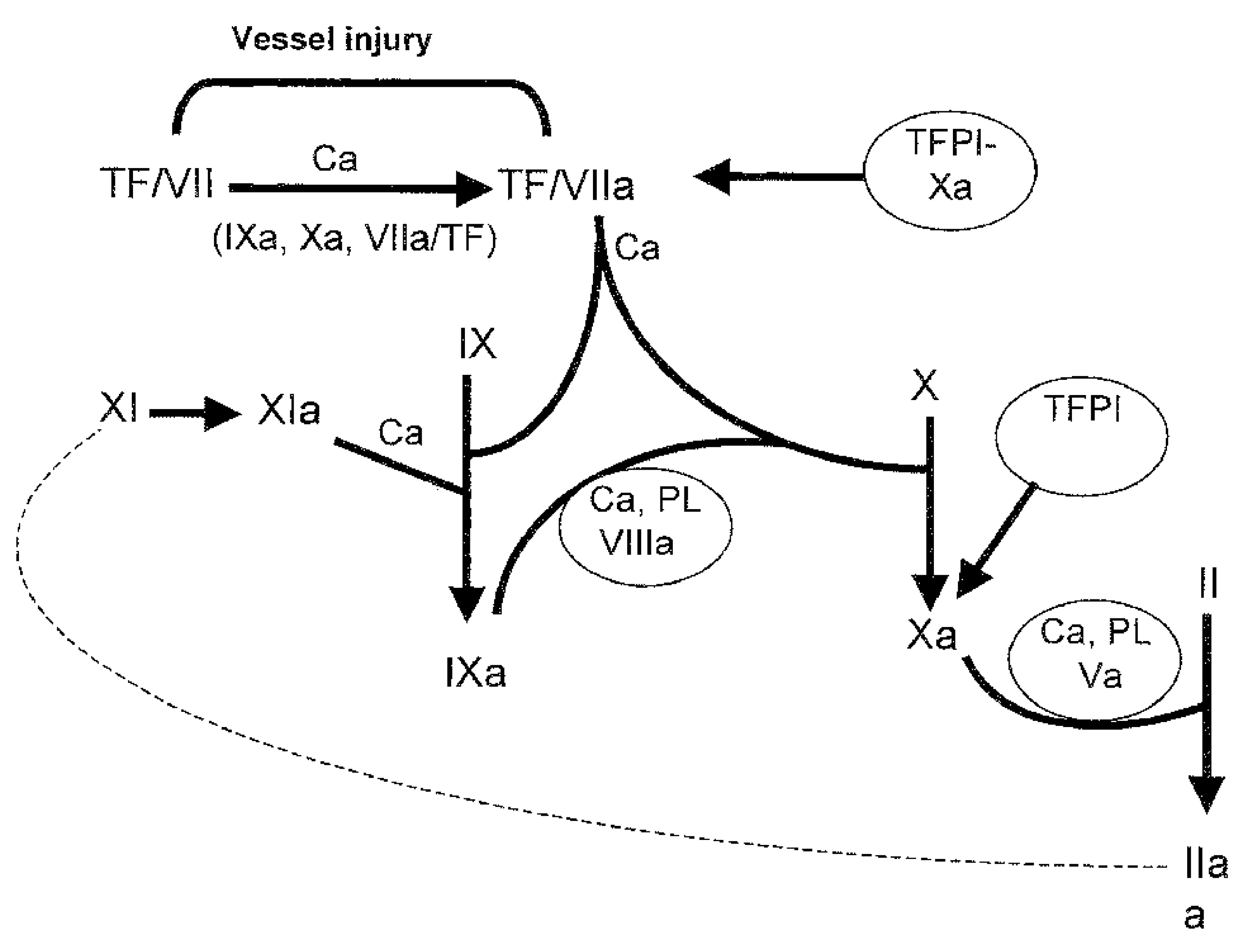
Primary Examiner — Alexander Kim

(74) *Attorney, Agent, or Firm* — Saliwanchik, Lloyd & Eisenschenk

(57) **ABSTRACT**

The present invention relates to improved human FVIII variants having at least one substitution in the A2 and/or C2 domain. The present invention also relates to their uses in the treatment of hemophilia A, particularly in patients with inhibitors.

16 Claims, 27 Drawing Sheets



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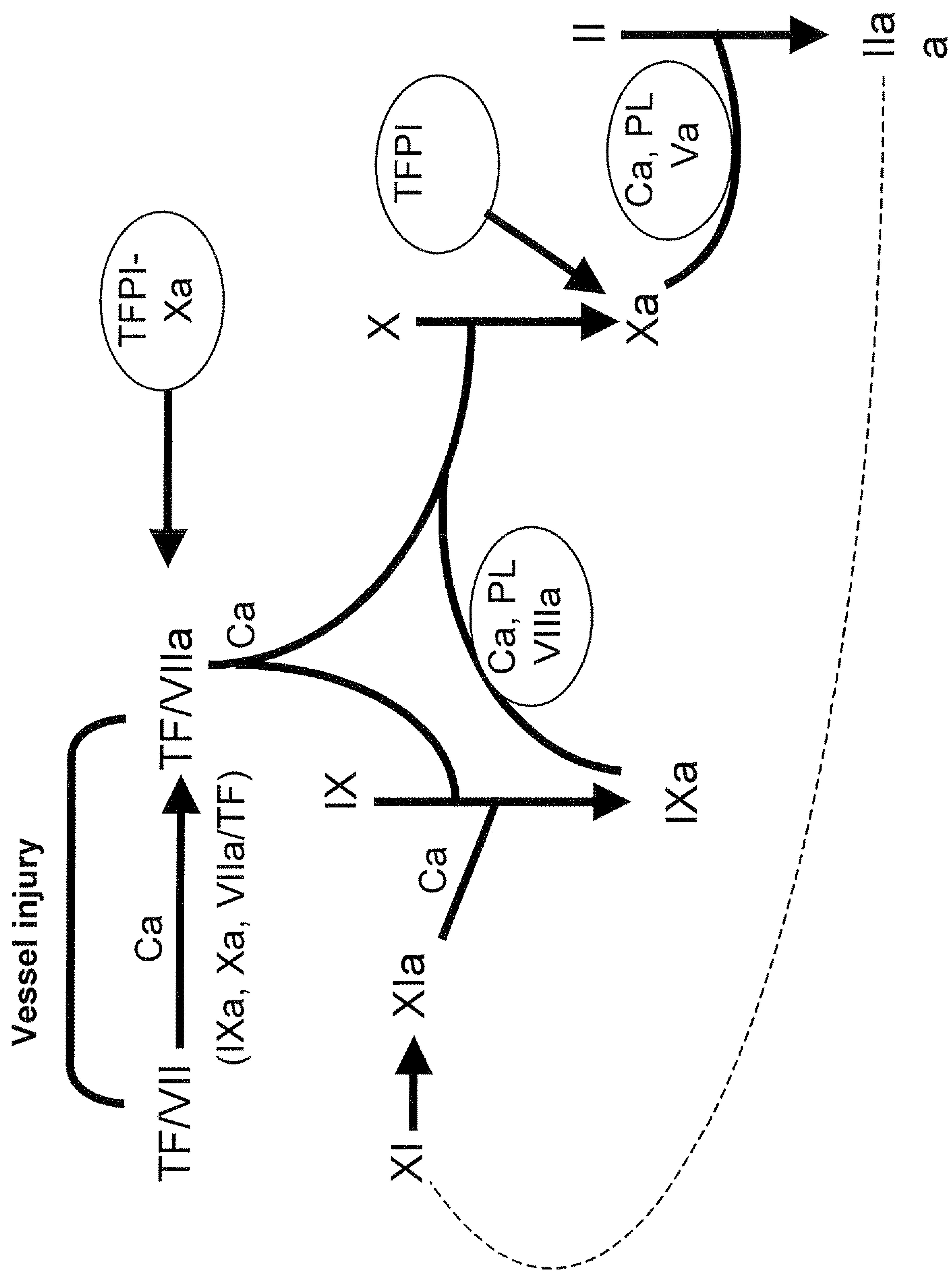
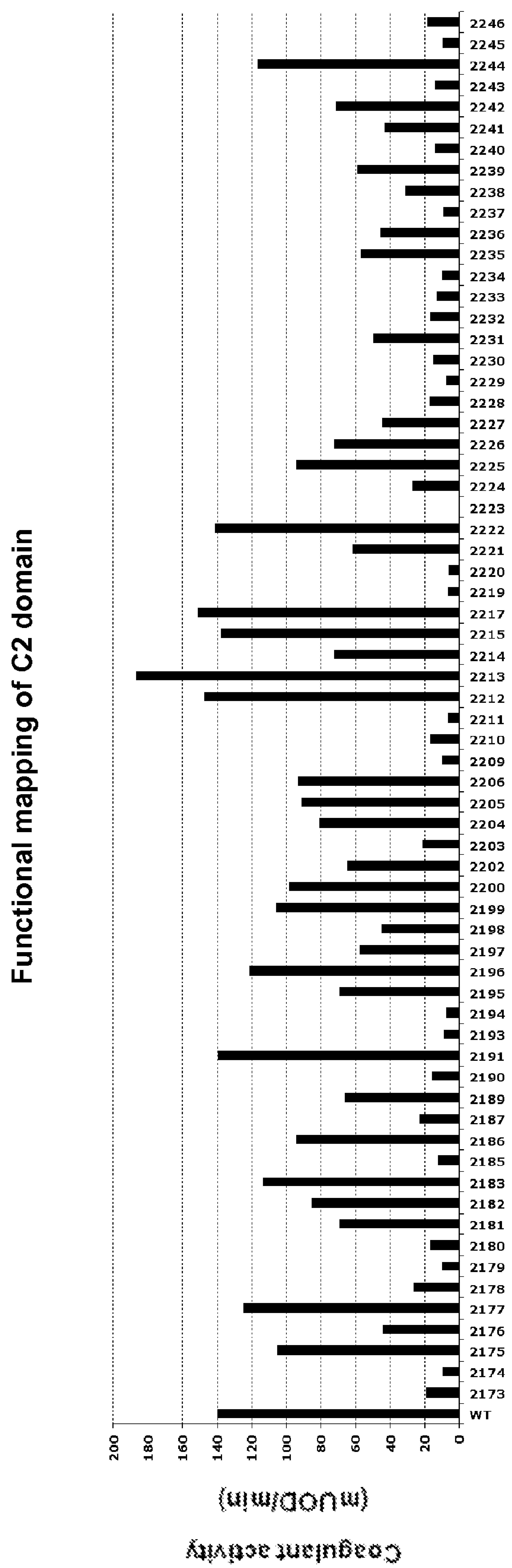


Vessel injury
TF/VII
Ca
TF/VIIa
(IXa, Xa, VIIa/TF)
TFPI-
Xa
Ca
IX
IXa
XI
XIa
Ca
Ca, PL
VIIIa
X
Xa
TFPI
Ca, PL
Va
II
IIa

FIGURE 1


Functional mapping of C2 domain
Coagulant activity
(mUOD/min)
0
20
40
60
80
100
120
140
160
180
200
WT
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2185
2186
2187
2189
2190
2191
2193
2194
2195
2196
2197
2198
2199
2200
2202
2203
2204
2205
2206
2209
2210
2211
2212
2213
2214
2215
2217
2219
2220
2221
2222
2223
2224
2225
2226
2227
2228
2229
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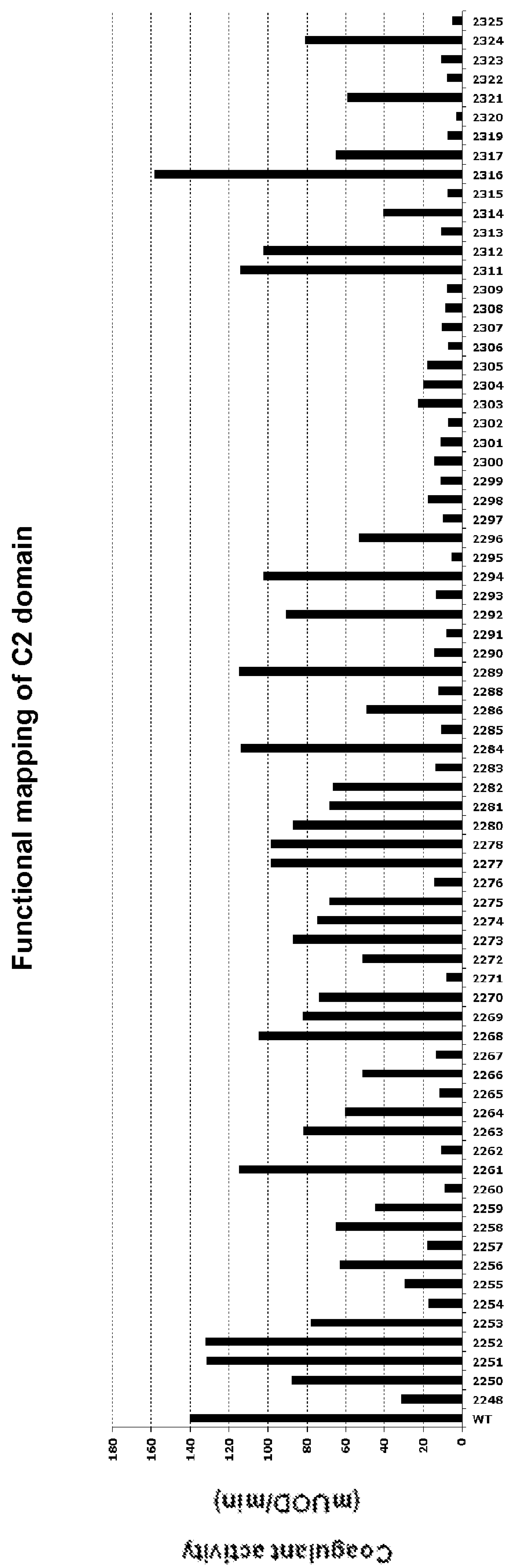
2232
2233
2234
2235
2236
2237
2238
2239
2240
2241
2242
2243
2244
2245
2246

FIGURE 2A


Functional mapping of C2 domain
Coagulant activity
(mUOD/min)
0
20
40
60
80
100
120
140
160
180
WT
2248
2250
2251
2252
2253
2254
2255
2256
2257
2258
2259
2260
2261
2262
2263
2264
2265
2266
2267
2268
2269
2270
2271
2272
2273
2274
2275
2276
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2282
2283
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2286
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2289
2290
2291
2292
2293
2294
2295
2296
2297
2298
2299
2300
2301
2302
2303
2304
2305
2306
2307
2308
2309
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2314
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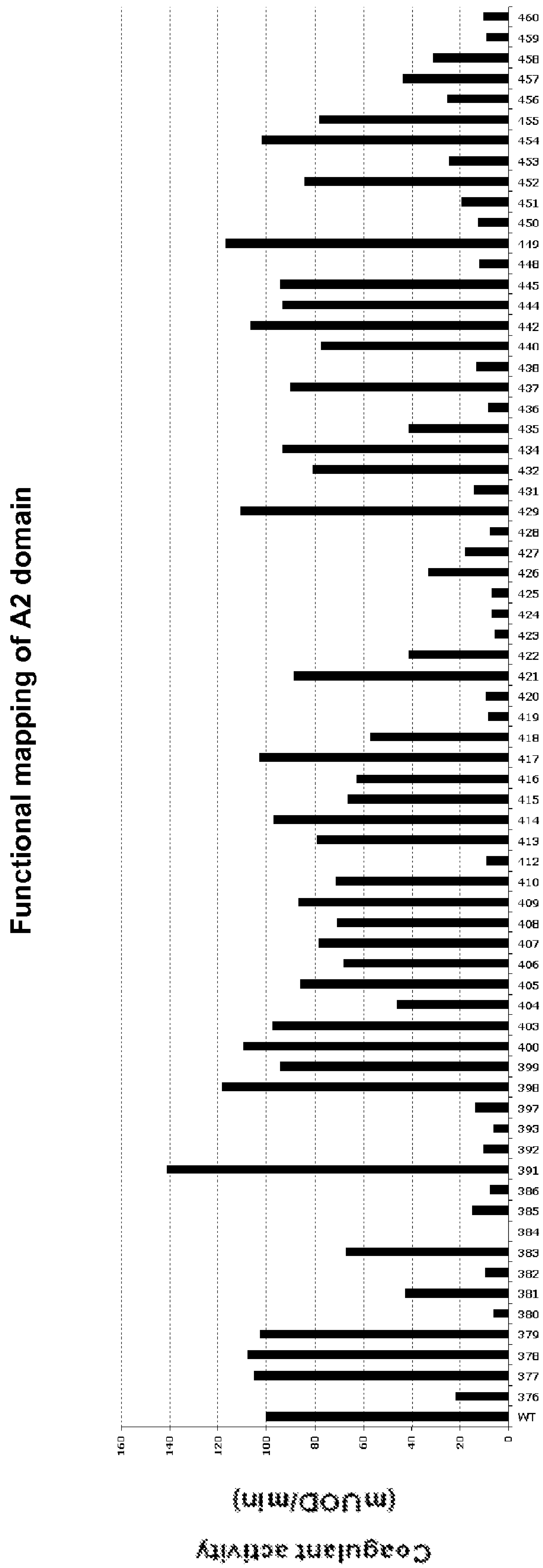
2316
2317
2319
2320
2321
2322
2323
2324
2325

FIGURE 2B



Functional mapping of A2 domain
Coagulant activity
(mUOD/min)
0
20
40
60
80
100
120
140
160
WT
376
377
378
379
380
381
382
383
384
385
386
391
392
393
397
398
399
400
403
404
405
406
407
408
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410
412
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421
422
423
424
425
426
427
428
429
431
432
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459
460

FIGURE 2C

Functional mapping of A2 domain

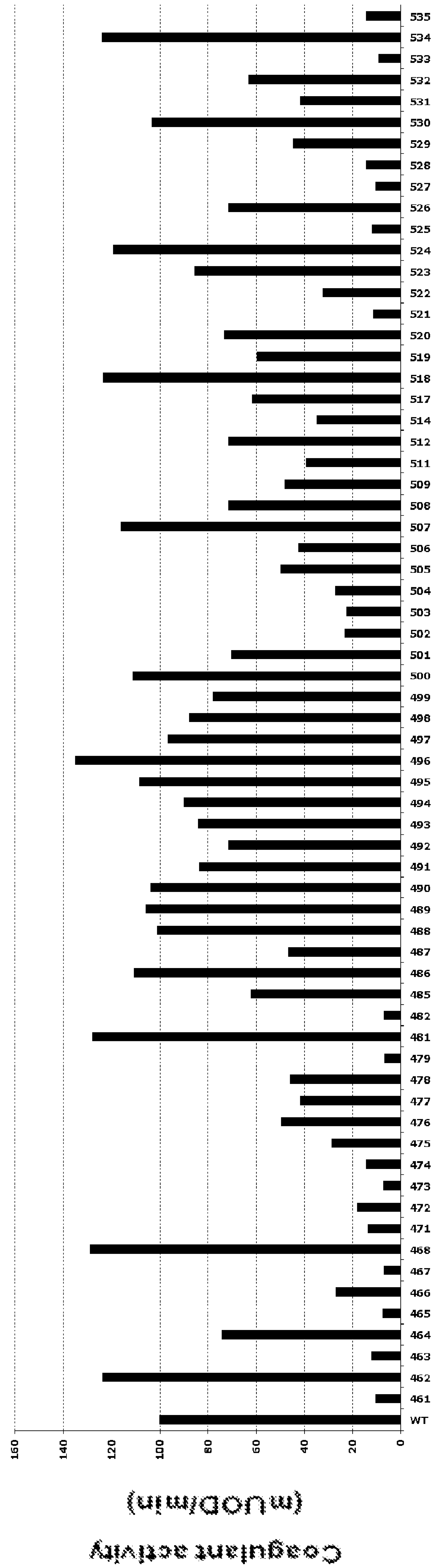


FIGURE 2D

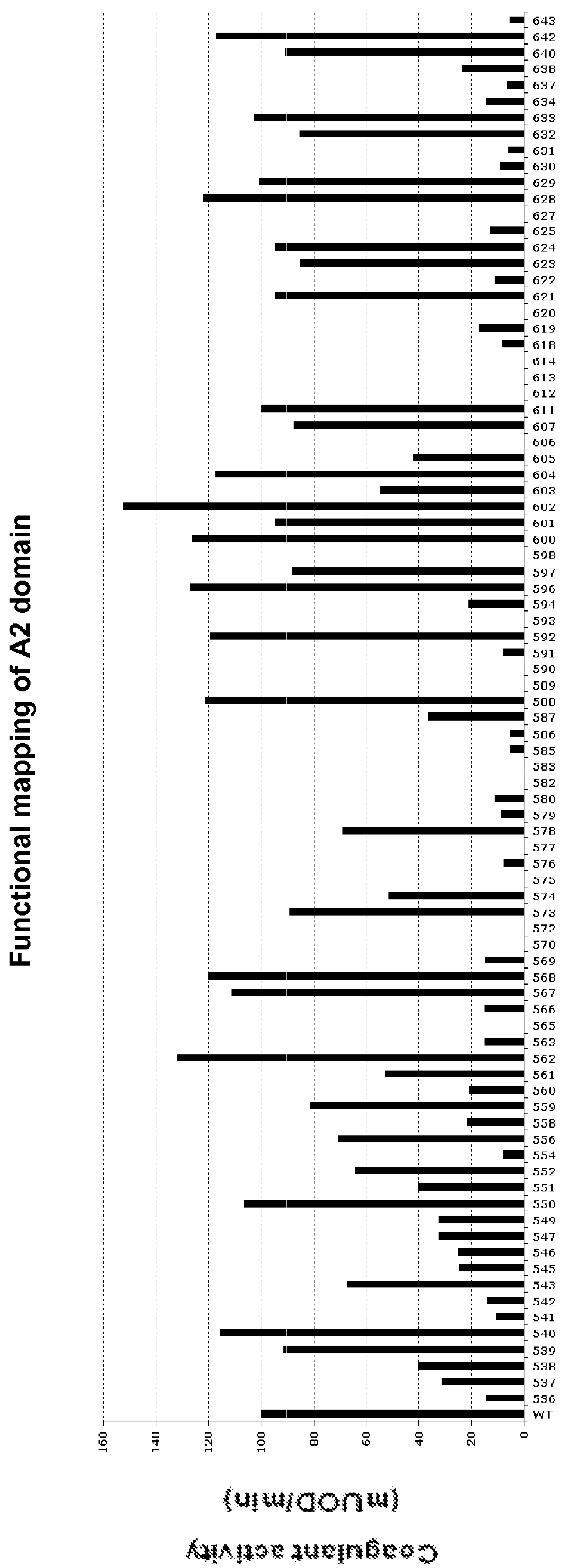

Functional mapping of A2 domain
Coagulant activity
(mUOD/min)
0
20
40
60
80
100
120
140
160
WT
536
537
538
539
540
541
542
543
545
546
547
549
550
551
552
554
556
558
559
560
561
562
563
565
566
567
568
569
570
572
573
574
575
576
577
578
579
580
582
583
585
586
587
588
589
590
591
592
593
594
596
597
598
600
601
602
603
604
605
606
607
611
612
613
614
618
619
620
621
622
623
624
625
627
628
629
630
631
632
633
634
637
638
640
642
643

FIGURE 2E

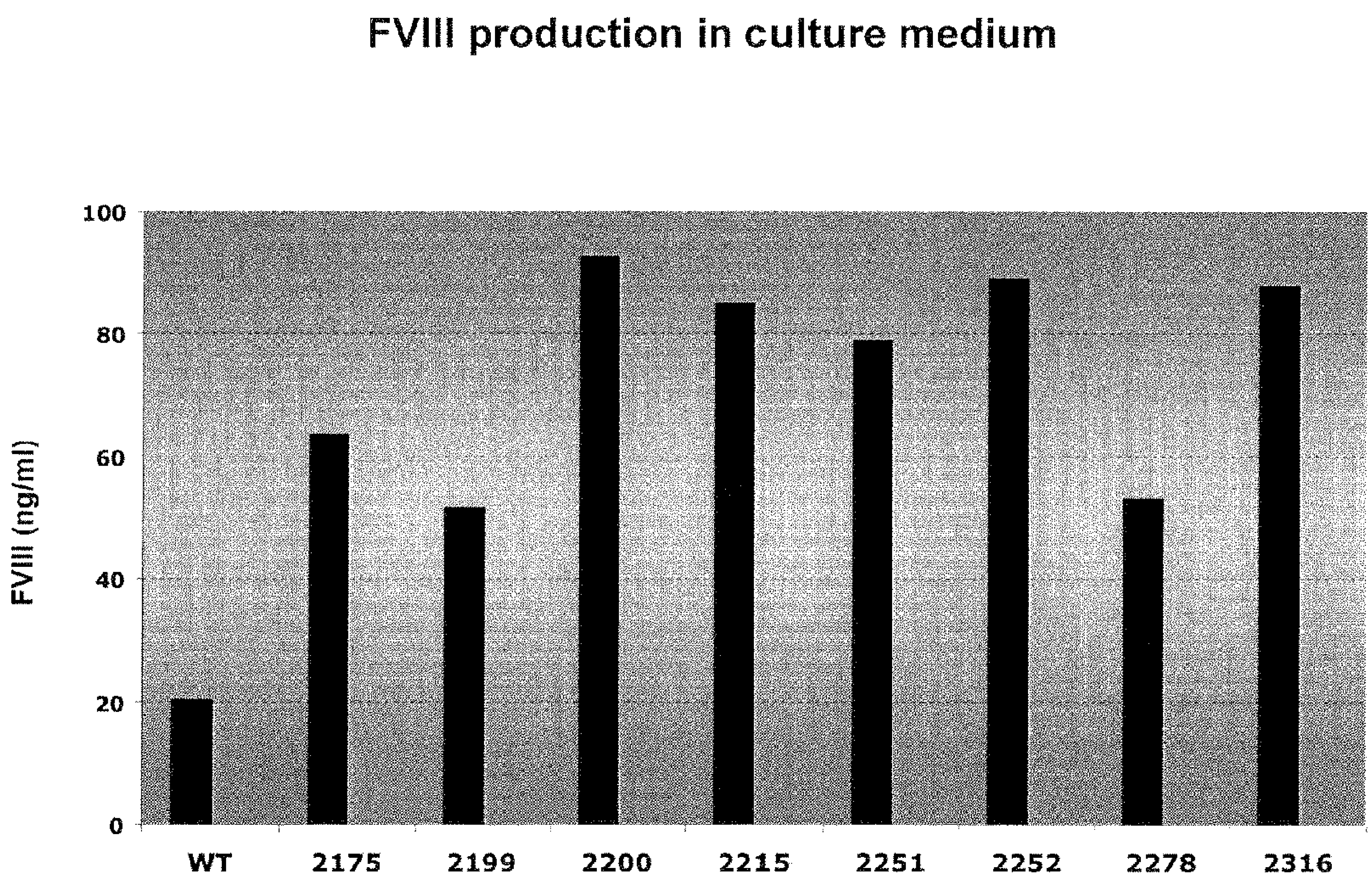
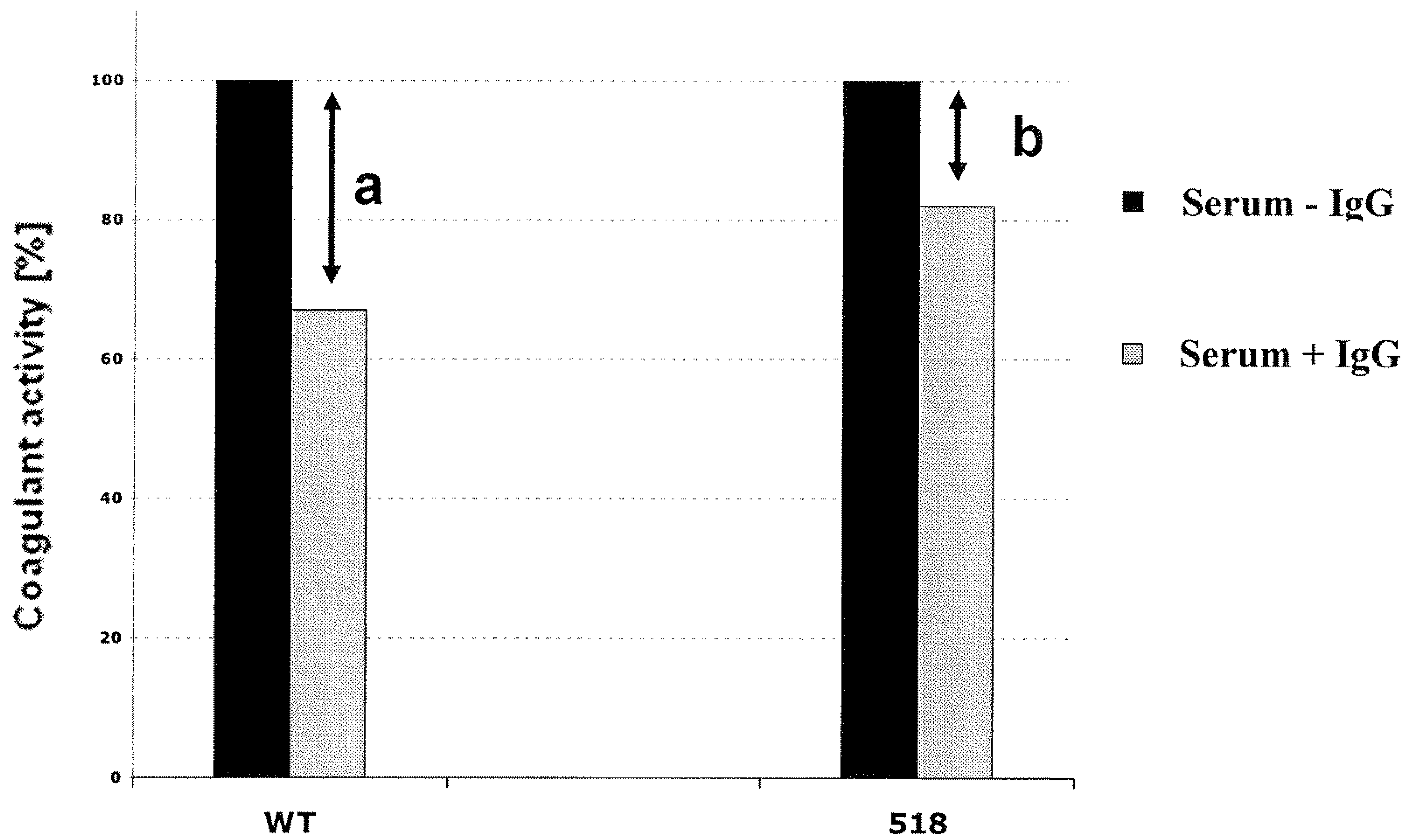

FVIII production in culture medium
FVIII (ng/ml)
100
80
60
40
20
0
WT
2175
2199
2200
2215
2251
2252
2278
2316

FIGURE 3

C2 mutants	Concentration higher than 10 ng/ml	Specific activity higher than 4 (mUOD/min/ng/ml)
2177	18,40	6,77
2183	11,80	9,57
2186	17,62	5,33
2191	27,27	5,11
2196	28,59	4,24
2204	14,36	5,62
2205	11,39	7,99
2206	14,62	6,36
2213	25,49	7,34
2217	24,75	6,11
2235	13,35	4,27
2258	11,61	5,60
2264	11,23	5,37
2268	11,29	9,24
2269	16,38	5,01

FIGURE 4

Example of abolition to inhibition of mutant 518 on serum of patient TD



a = % residual activity (WT)
b = % residual activity (mutant)
% abolition to inhibition = -[(b-a) / a] x 100

FIGURE 5

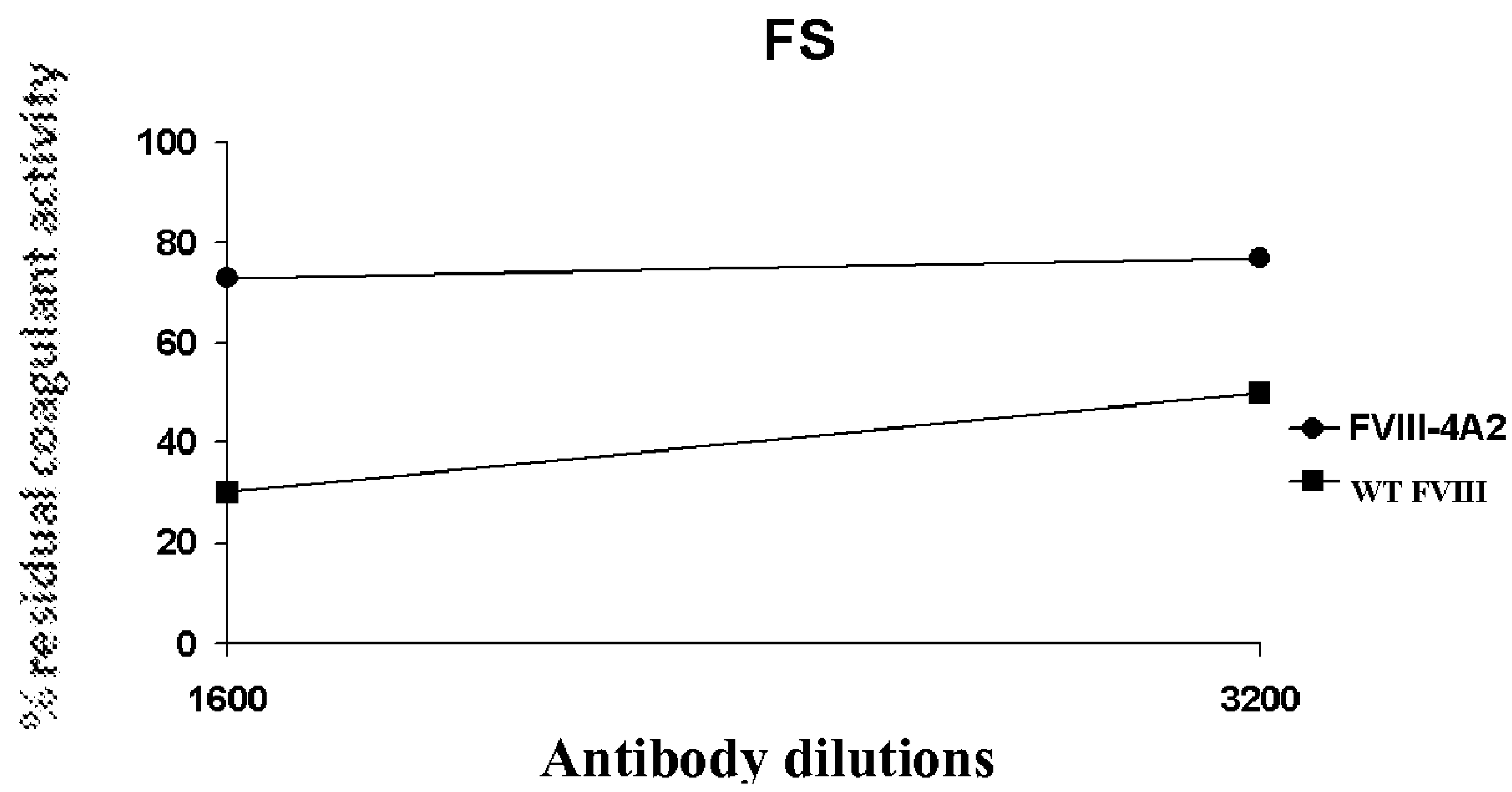


FIGURE 6A

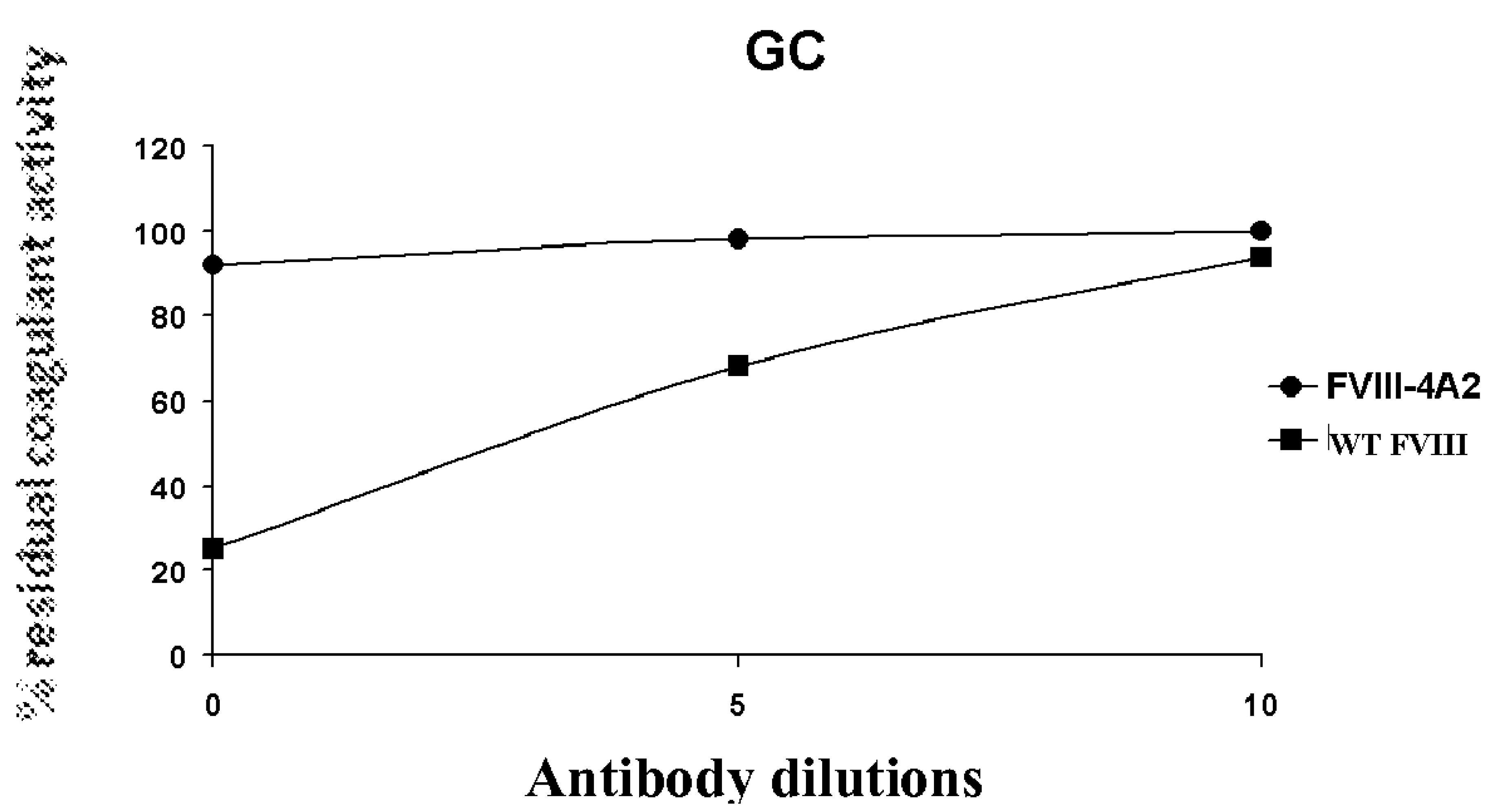


FIGURE 6B

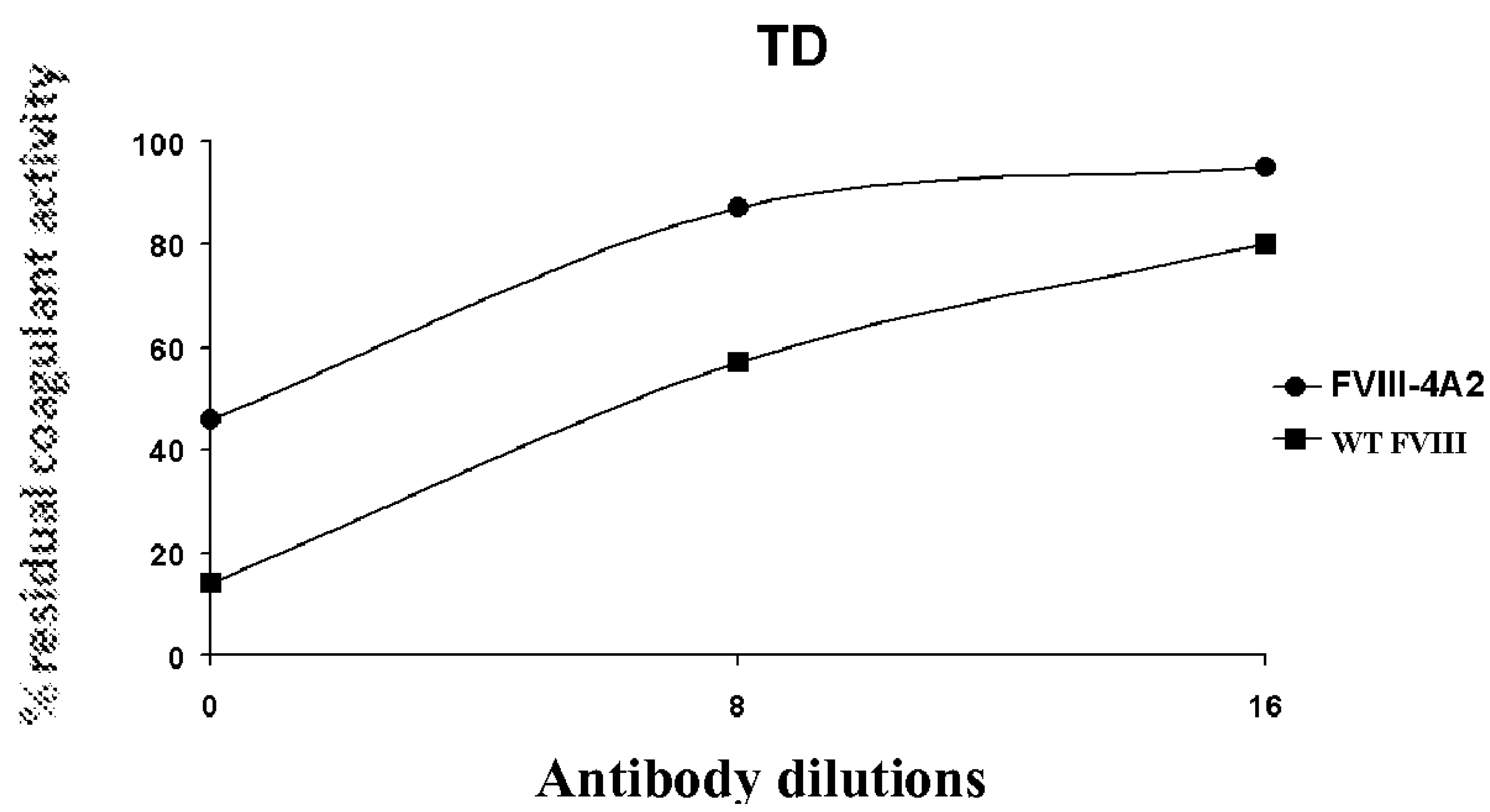


FIGURE 6C

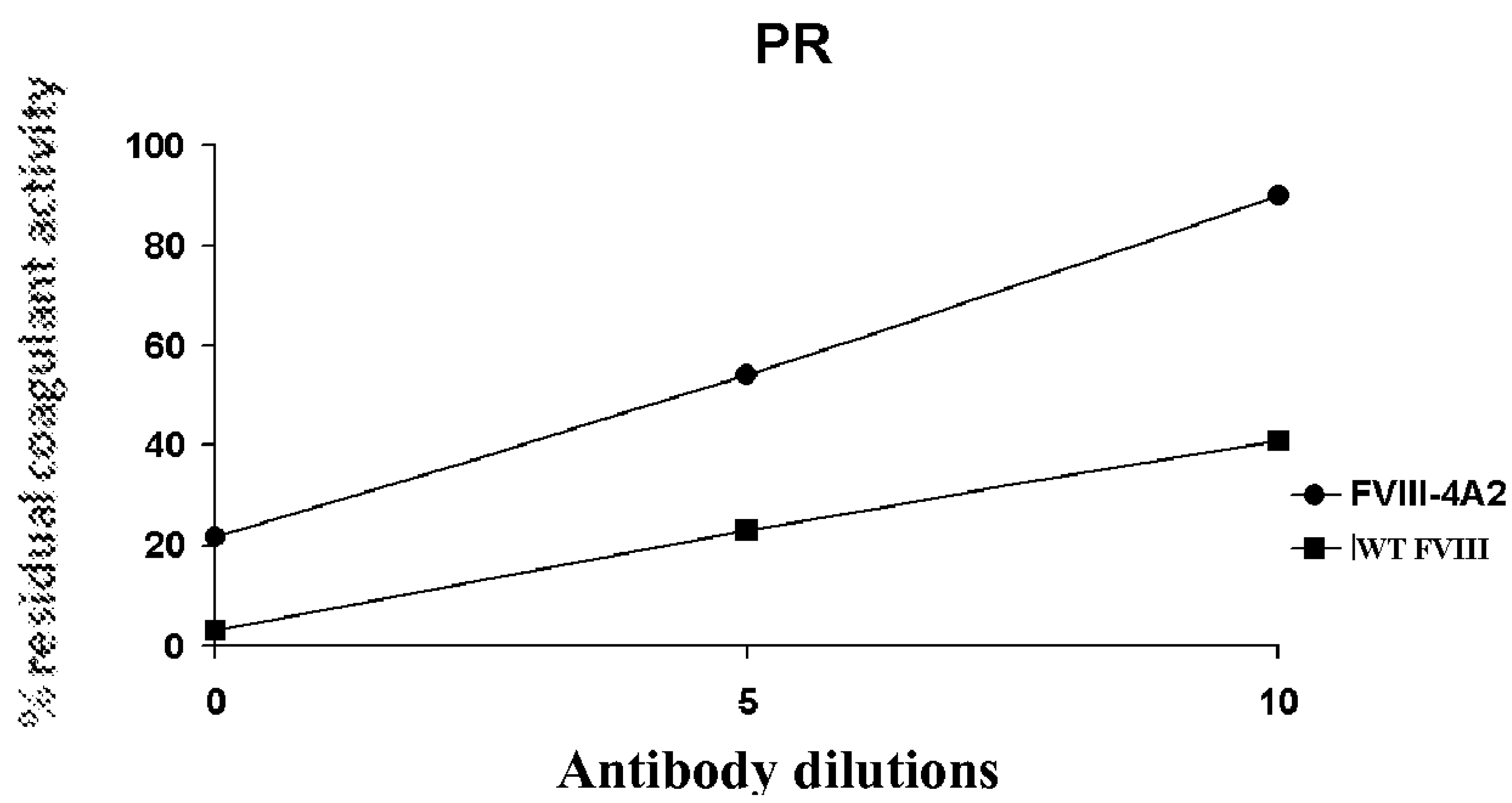


FIGURE 6D

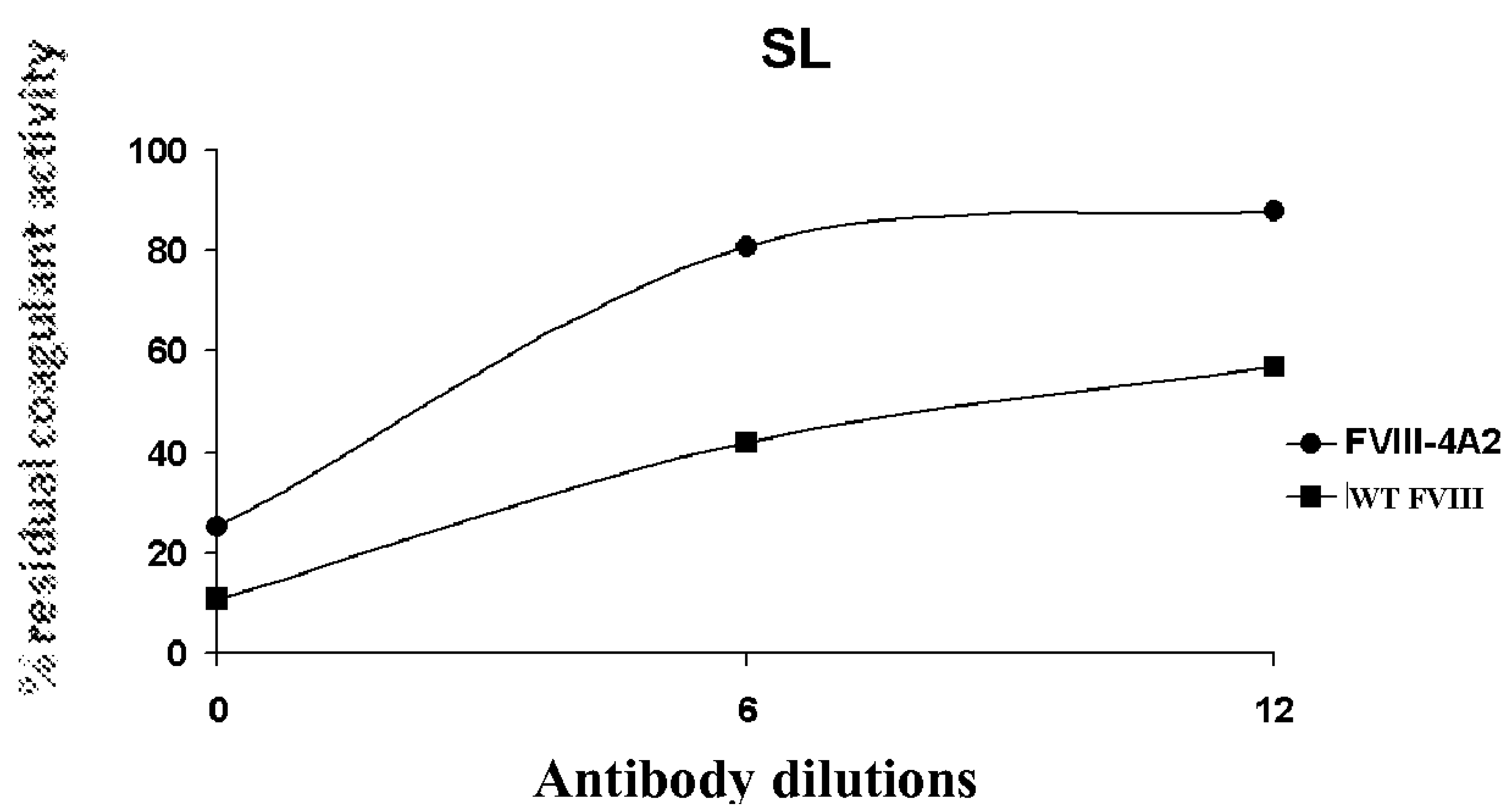


FIGURE 6E

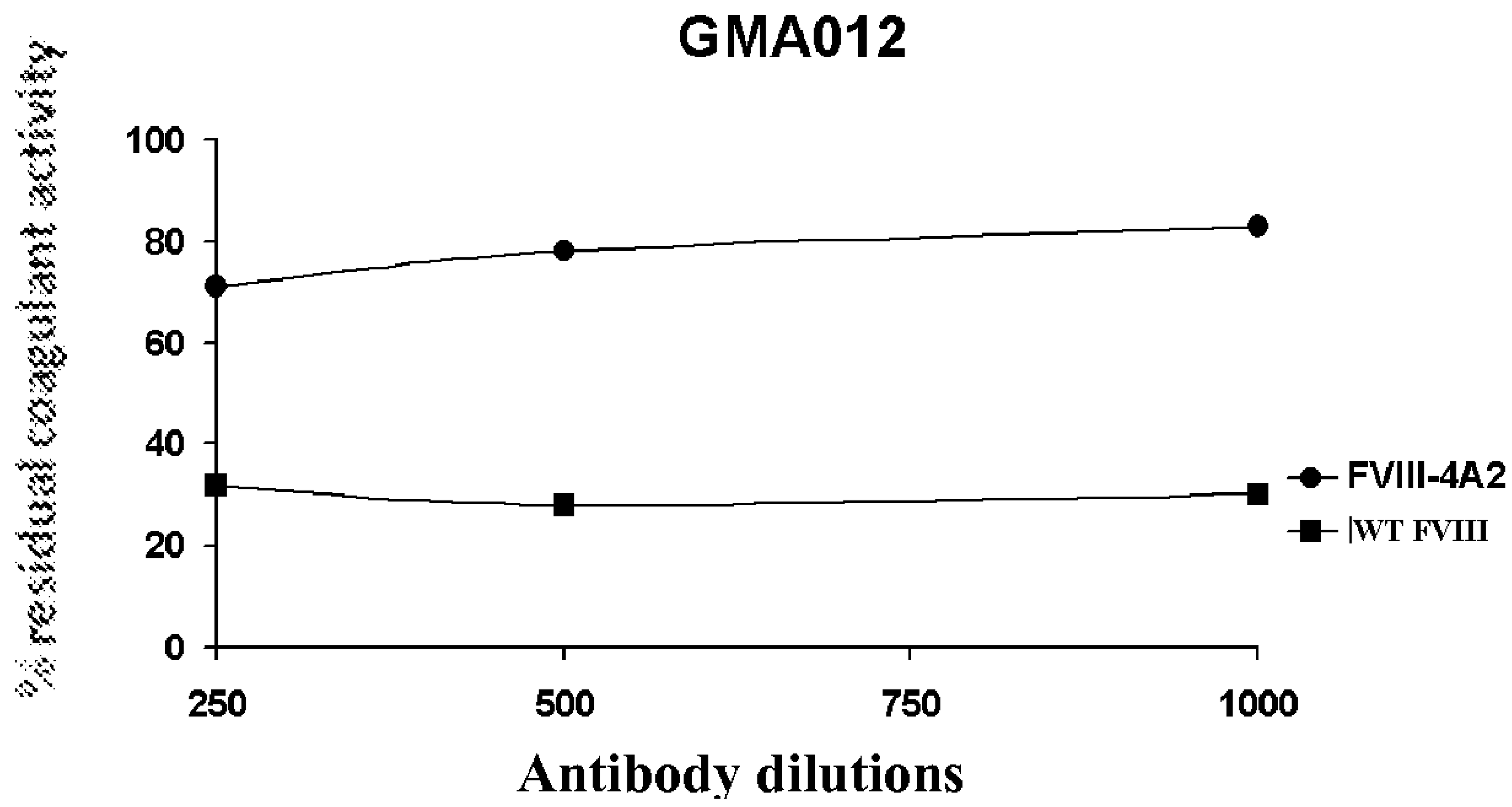


FIGURE 7A

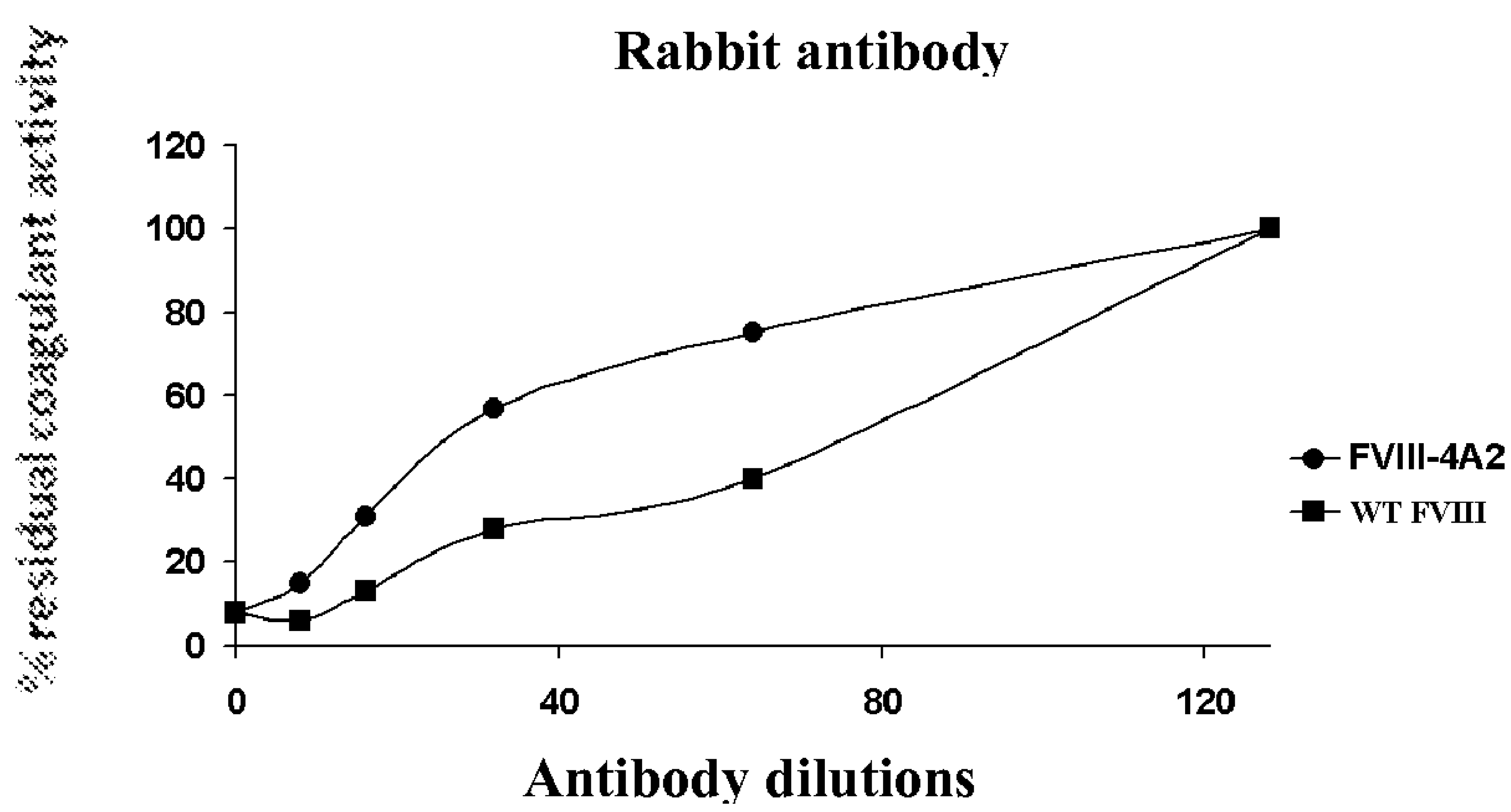


FIGURE 7B

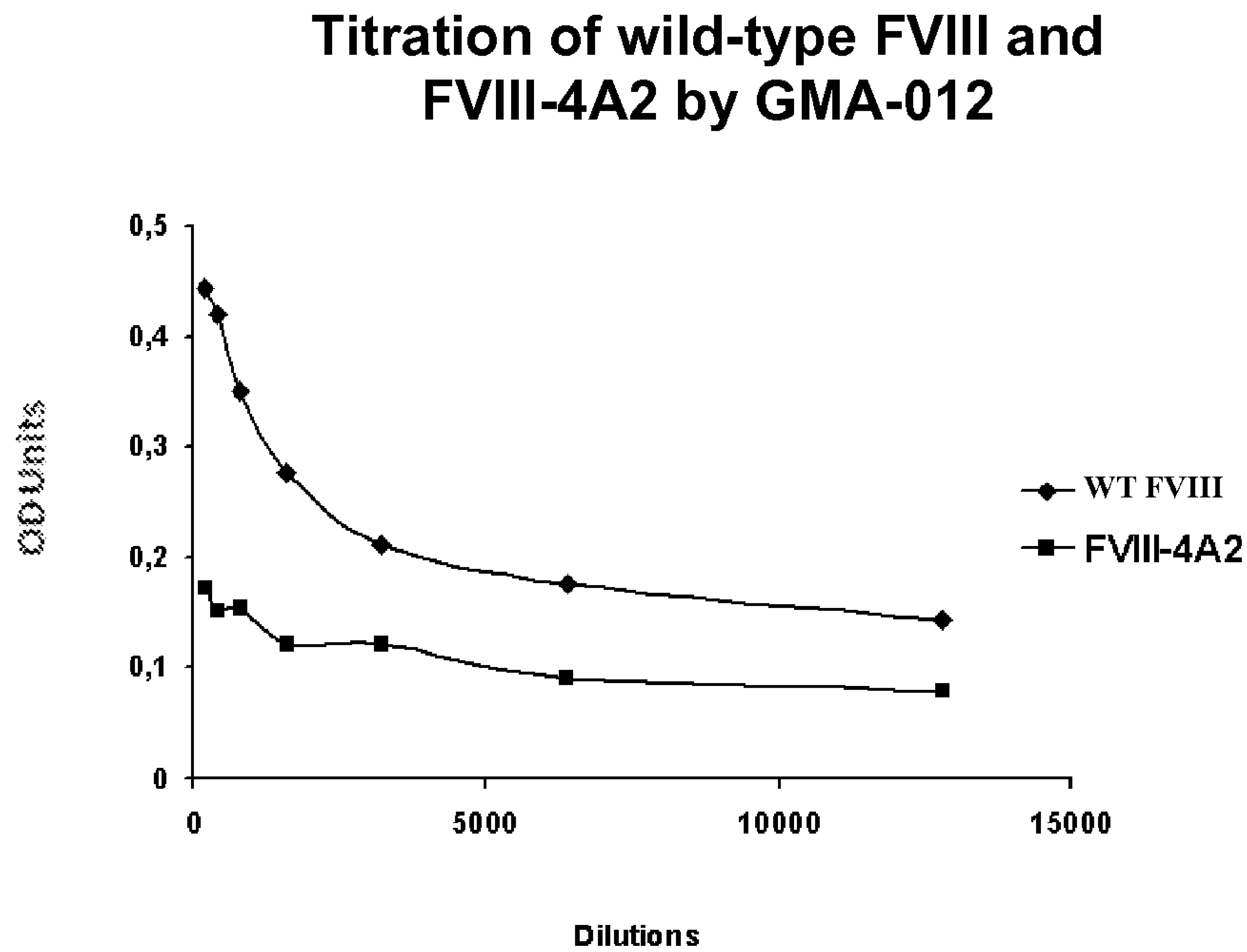


FIGURE 8A

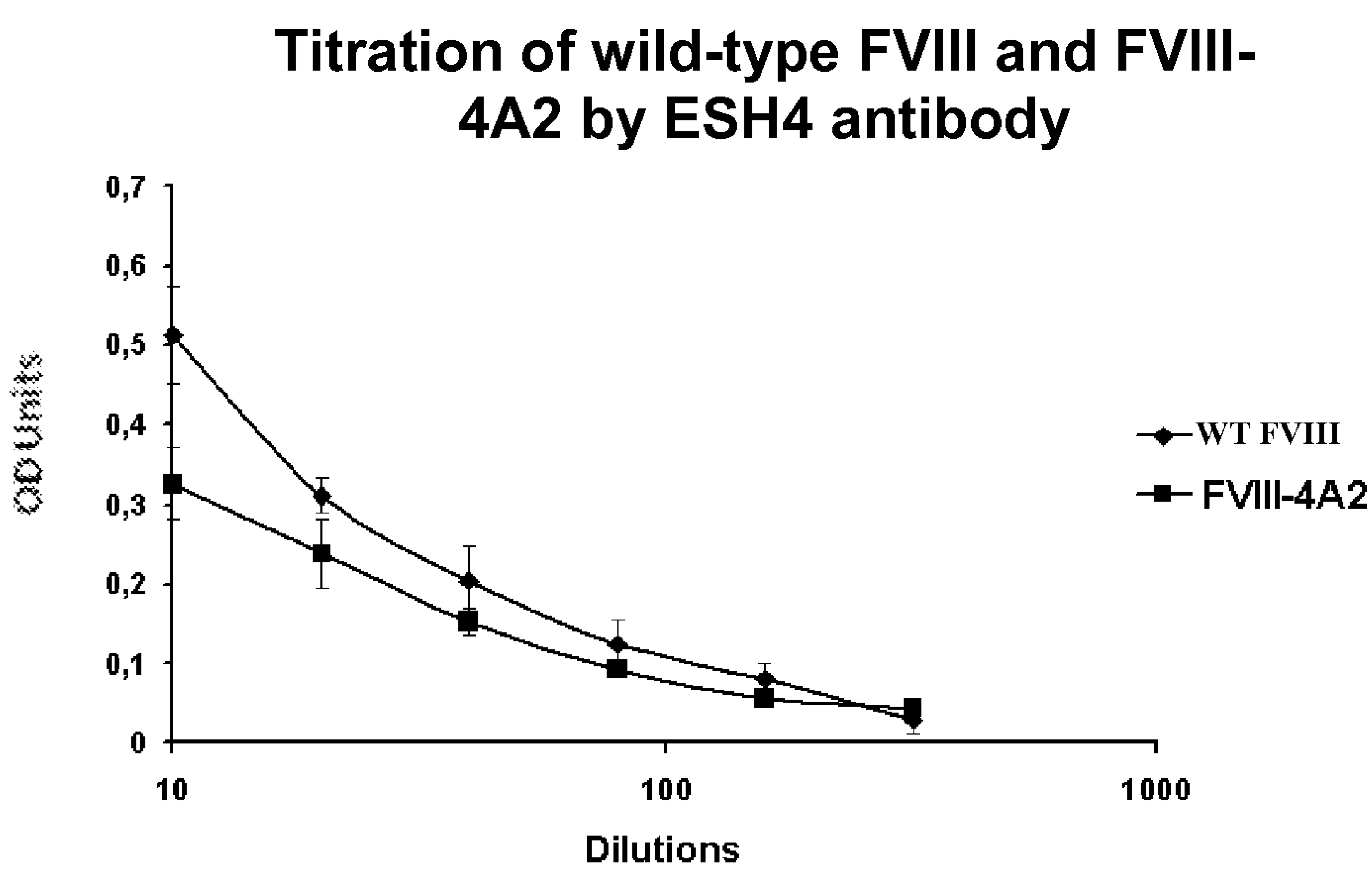


FIGURE 8B

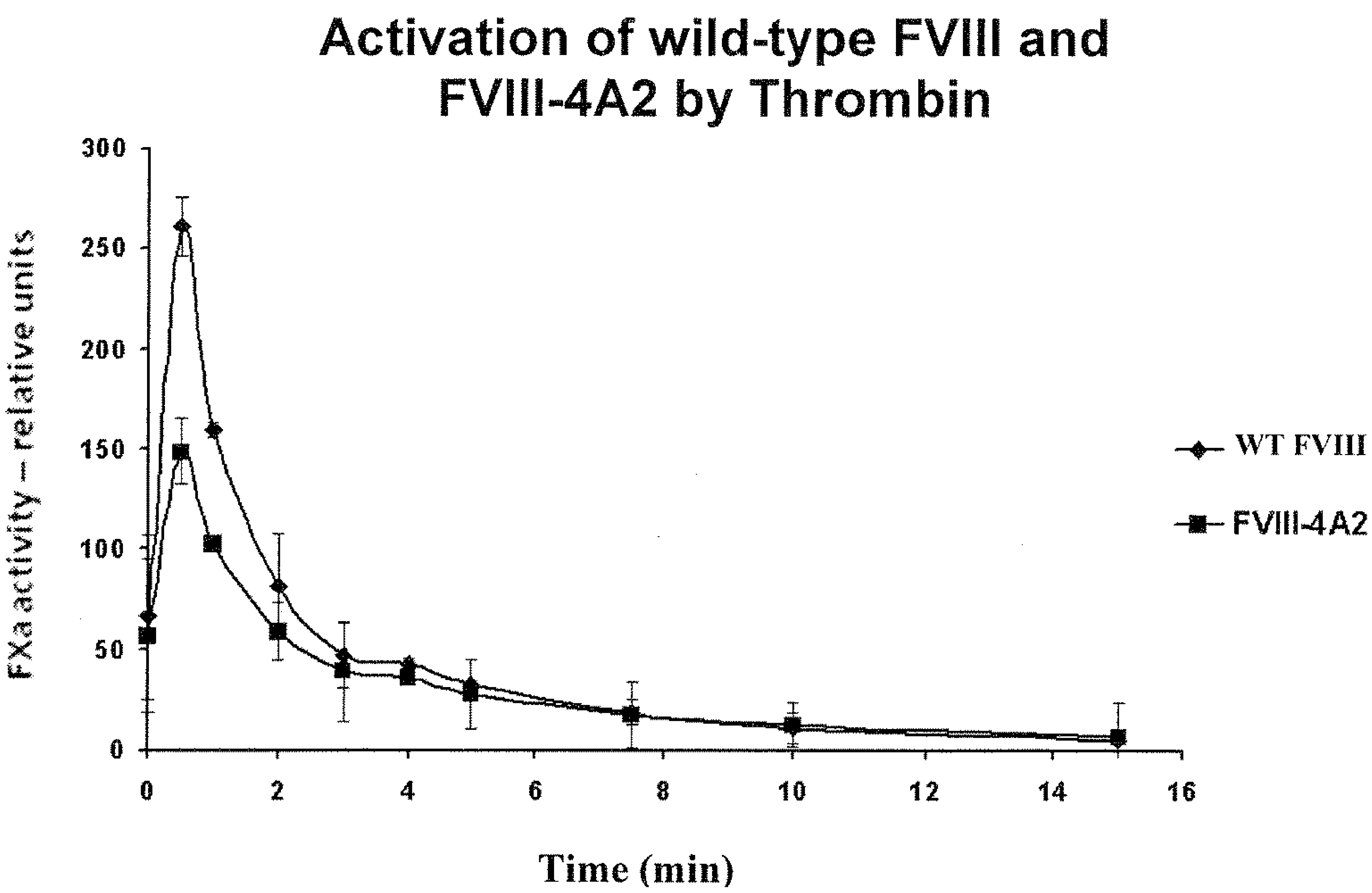

Activation of wild-type FVIII and FVIII-4A2 by Thrombin
FXa activity – relative units
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
Time (min)
WT FVIII
FVIII-4A2

FIGURE 9

Loss of procoagulant activity after activation of wild-type FVIII and FVIII-4A2 by IIa

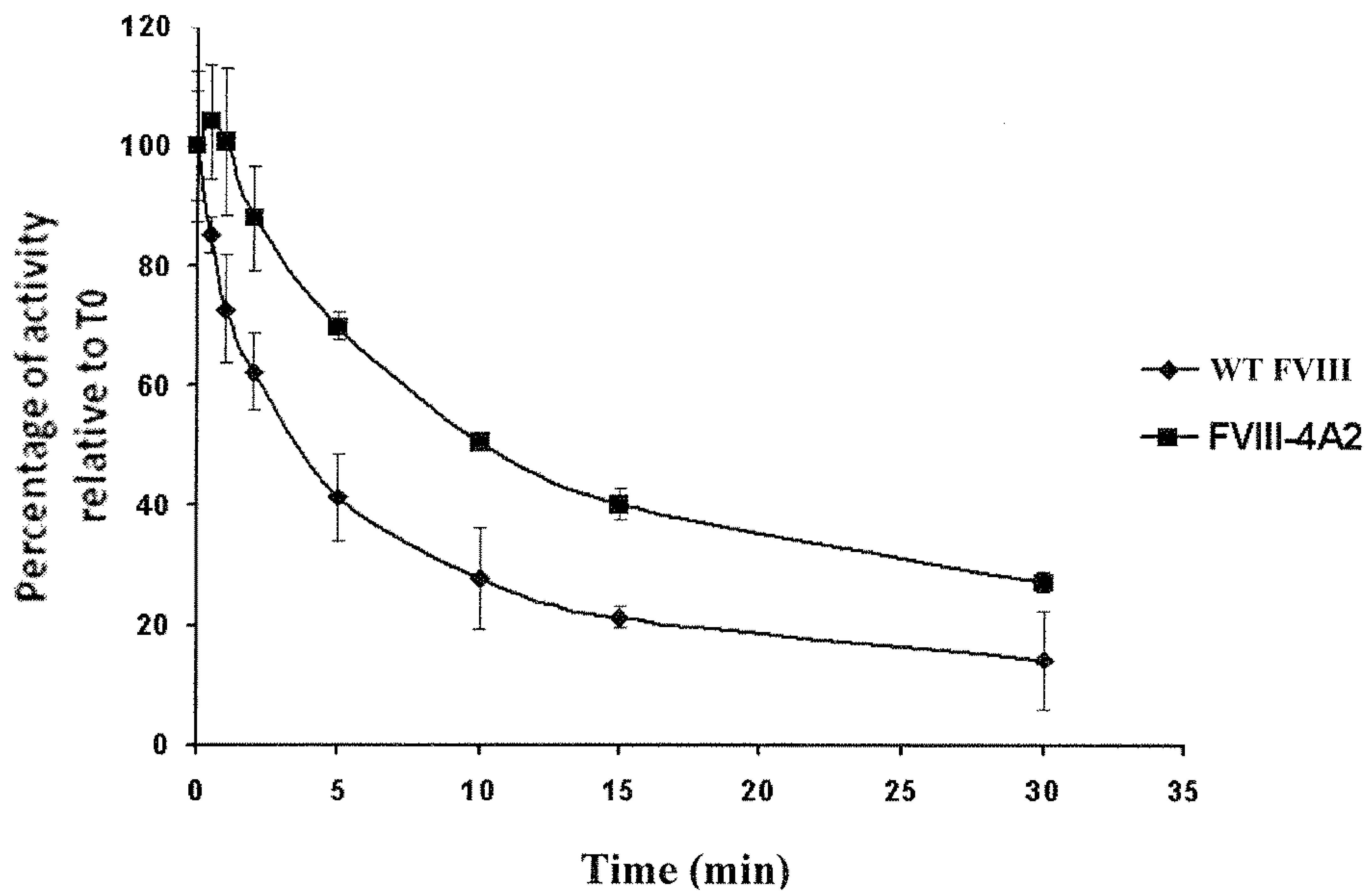


FIGURE 10

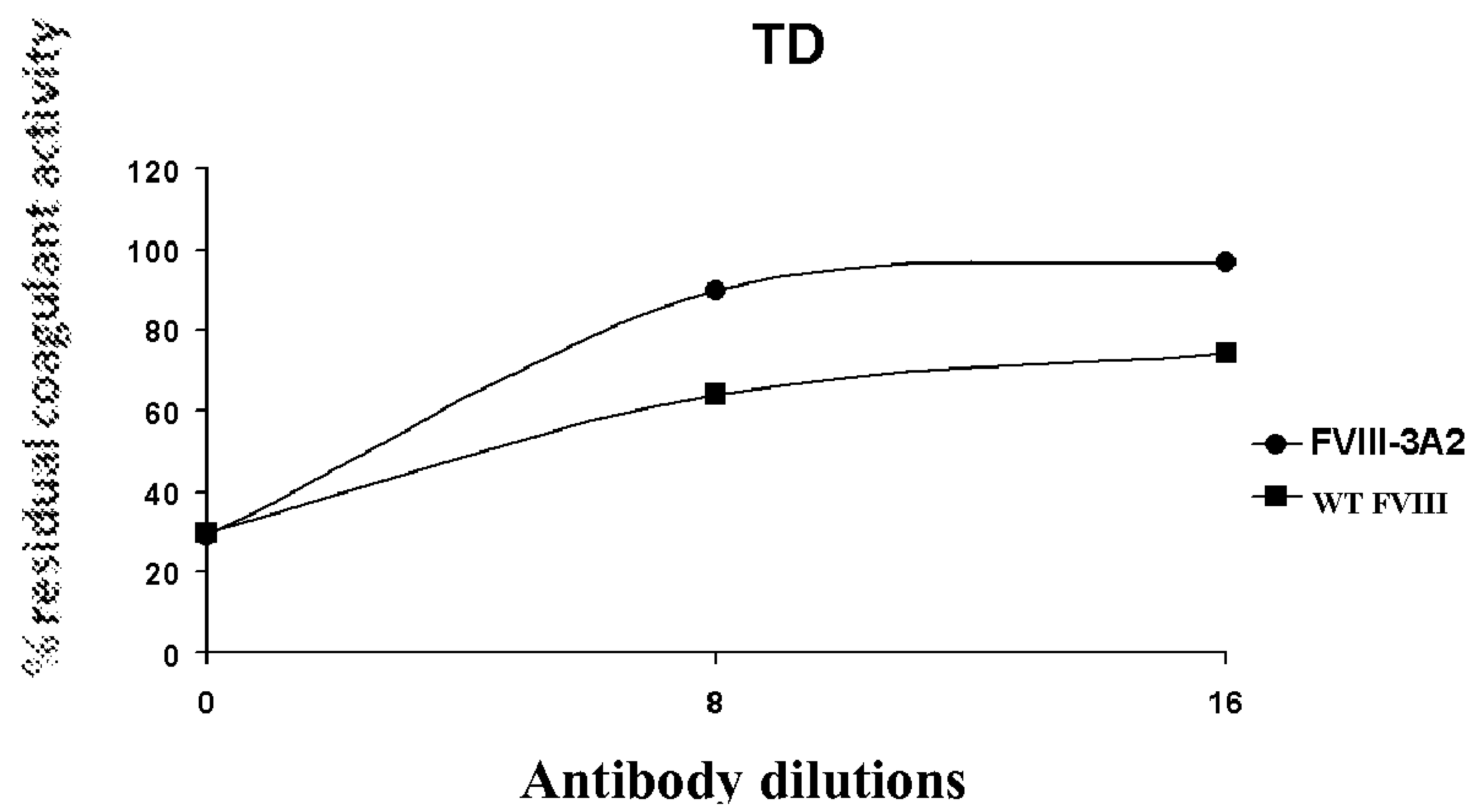


FIGURE 11A

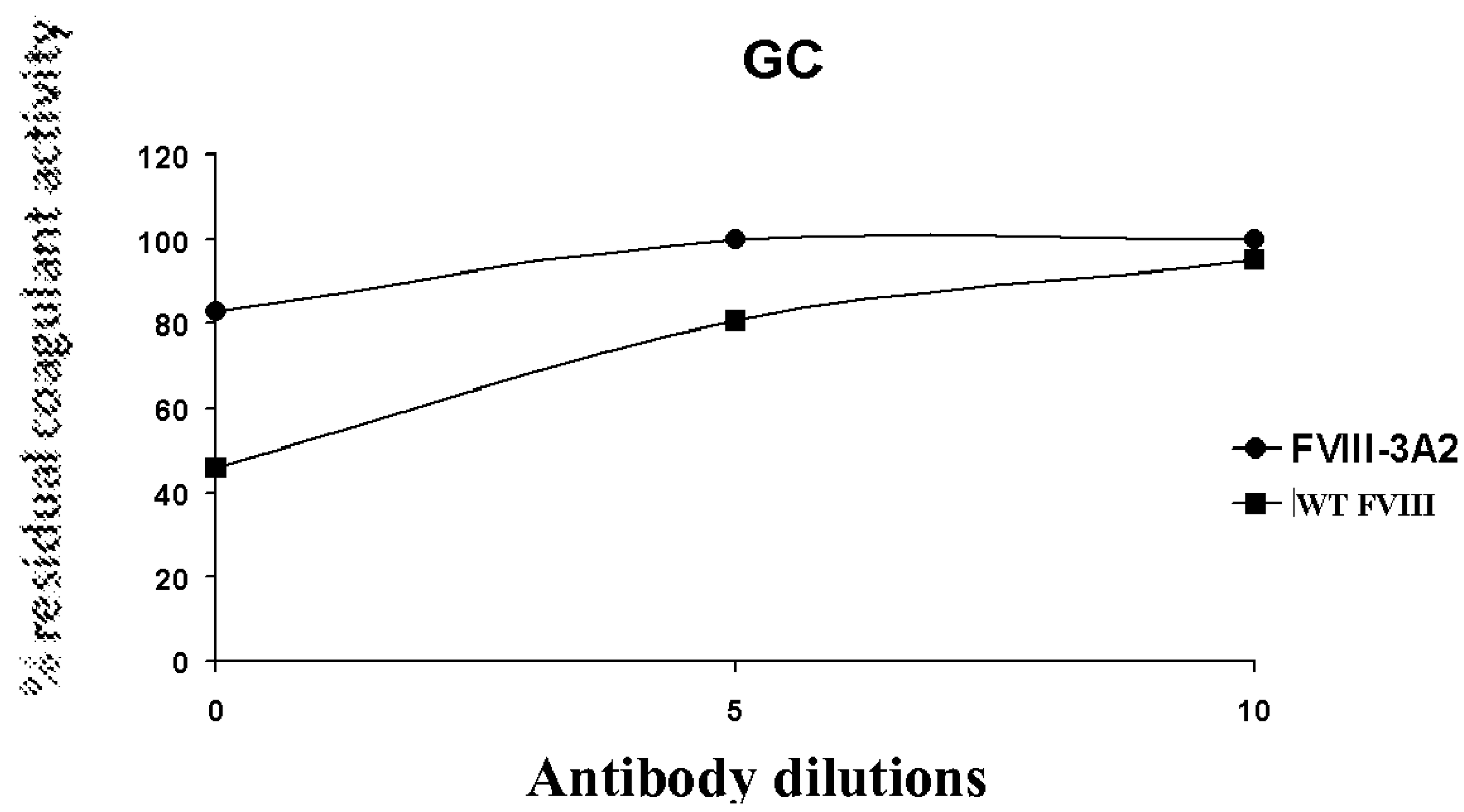


FIGURE 11B

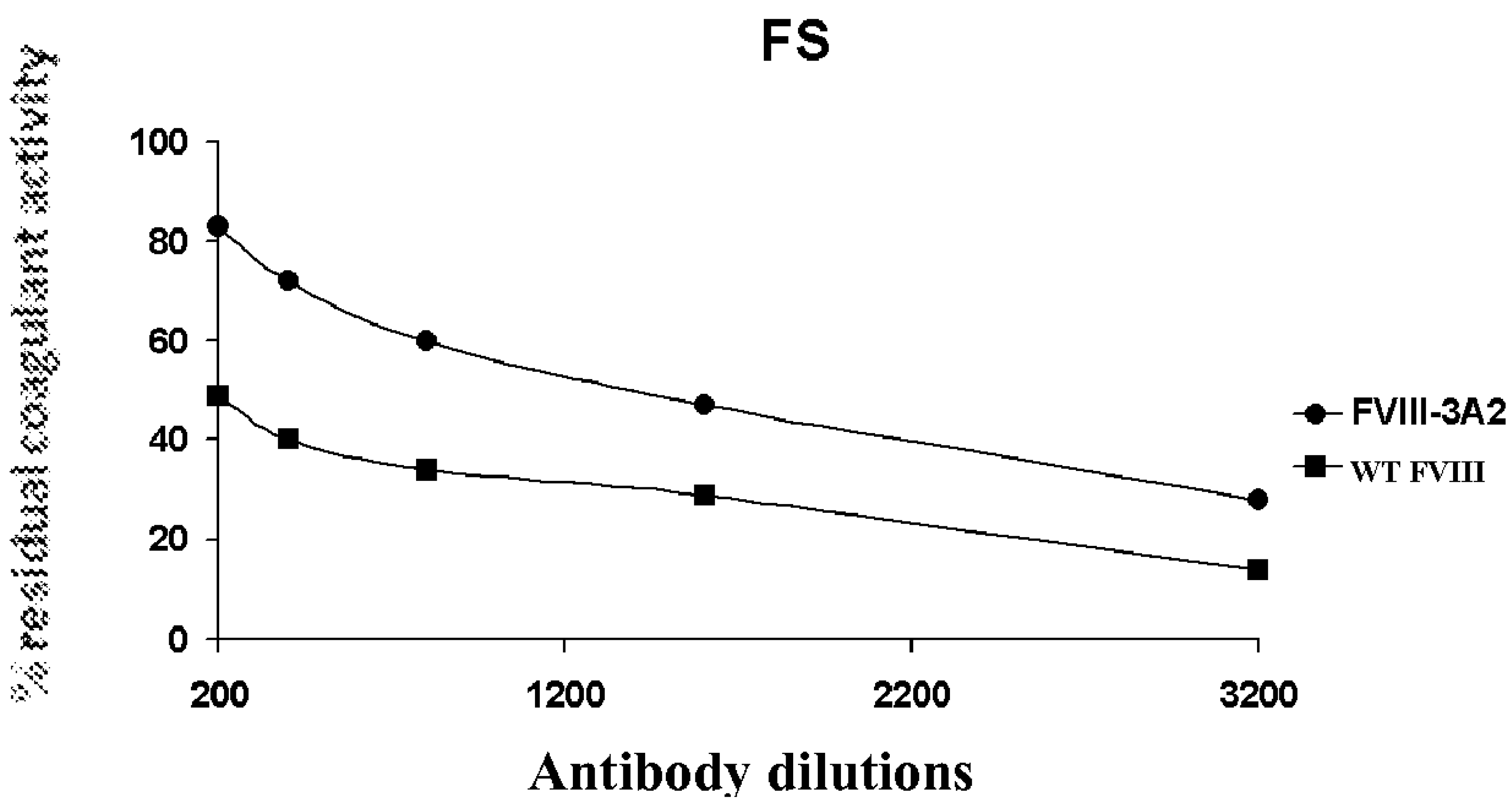


FIGURE 11C

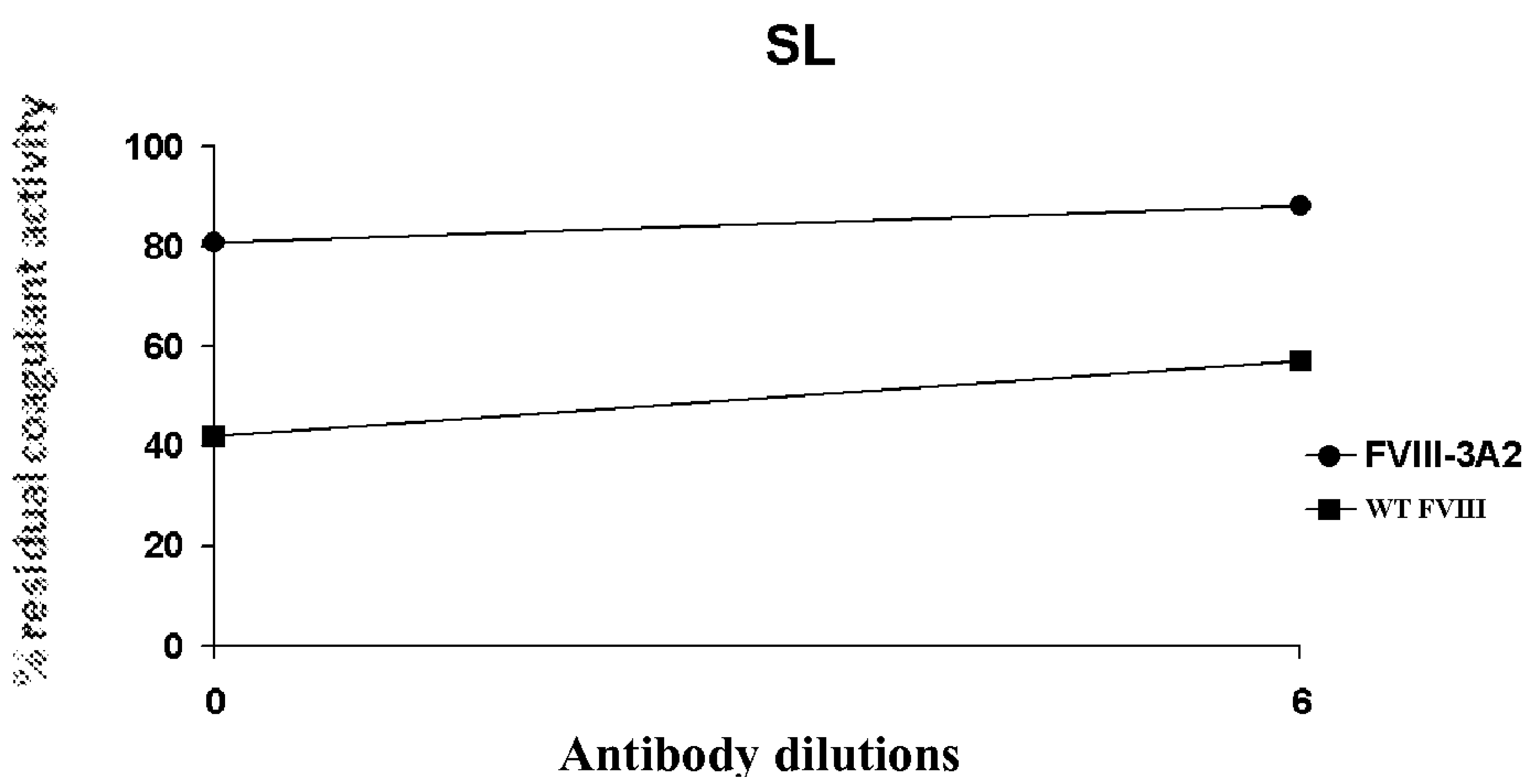


FIGURE 11D

mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)
2175	103,5	2215	215	2269	98	2324	85
2177	125,5	2217	162,5	2270	123,5		
2181	81,6	2221	71,5	2273	126,5	WT	100
2182	85,3	2222	149,5	2274	88		
2183	120,5	2225	85,5	2275	81,5		
2186	80,5	2226	81	2277	151,5		
2189	74,8	2235	72,5	2278	133		
2191	175,6	2239	76	2280	116,5		
2195	86,8	2242	90,5	2281	88,2		
2196	152,5	2244	124,5	2282	75,5		
2197	71	2250	126,5	2284	114		
2199	114,5	2251	131	2289	162		
2200	131,4	2252	136,5	2292	136		
2202	75,3	2253	86,5	2294	113		
2204	92	2256	76	2296	52,8		
2205	78	2258	97,5	2311	137		
2206	105,5	2261	174,5	2312	129,5		
2212	134	2263	126,5	2316	162,5		
2213	141	2264	115	2317	64		
2214	121	2268	116,5	2321	58		

FIGURE 12

mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)
377	110,5	421	128	489	142,5	523	92
378	147,5	429	113	490	121,5	524	131,5
379	114,5	432	100,5	491	100,5	526	73
383	67	434	82	492	113,5	530	109
391	153	437	82	493	78,5	532	63
398	88,5	440	72,5	494	101	534	138,5
399	94,5	442	96	495	131,5	539	91
400	132,5	444	91,5	496	143	540	137,5
403	101,5	445	96	497	121	543	67,2
405	86	449	128,5	498	133	550	114
406	148	452	99	499	78	552	64,2
407	78,5	454	140	500	126	556	85
408	78	455	87	501	125,5	559	145
409	138	462	128,5	507	117,5	562	157
410	71	464	81	508	86	567	115,5
413	104,5	468	178	512	71,5	568	136,5
414	113,5	481	172,5	517	61,5	573	93
415	66,5	485	62	518	152,5	578	83
416	62	486	147	519	60	588	145
417	118	488	148,5	520	80,5	592	165

FIGURE 13

mutant	Activity (mUOD/min)
596	147,5
597	87
600	132
601	99,5
602	157,5
604	146,5
607	106
611	125,5
621	108,5
623	128,5
624	128,5
628	123,5
629	107,5
632	110
633	113
640	146
642	134,5

FIGURE 14

		FS	TD	GC	PR	SL
Mutants	400	23	17	-	-	-
	486	14	24	10	14	-
	493	-	20	28	-	-
	403	34	-	10	-	16
	562	10	9	15	-	29
	414	33	9	-	-	-
	437	16	ND	-	-	-

FIGURE 15

		FS	TD	GC	PR	SL
Mutants	518	-	22	-	-	-
	2280	-	34	12	6	21
	2275	-	-	10	19	24
	2244	-	38	-	-	25
	2212	25	16	-	-	-
	2202	-	18	-	-	-

FIGURE 16

		FS	TD	GC	PR	SL
Mutants	421	33	9	5	-	-
	494	-	17	28	5	-
	496	-	-	24	15	16
	2206	21	30	-	-	5
	2226	-	32	3	-	-
	2261	17	-	-	-	5
	2281	-	22	-	3	6
	2282	-	30	-	3	-
	2311	-	35	13	-	-

FIGURE 17

		FS	TD	GC	PR	SL
Mutants	409	31	12	5	-	15
	462	25	12	5	-	28
	507	-	27	5	5	-
	629	-	40	-	12	15
	2312	-	36	-	-	-
	2289	-	30	12	-	13
	2316	-	46	10	-	36
	2294	-	28	36	-	20

FIGURE 18

mutant	Activité muDO/min	Activité Spécifique muDO/min/ng/ml	mutant	Activité muDO/min	Activité Spécifique muDO/min/ng/ml
2202	75,3	7,52	400	132,5	13,6
2206	105,5	7,25	403	101,5	7,05
2212	134	6,89	409	138	12,35
2226	81	12,6	414	113,5	14,34
2244	124,5	10,02	421	128	19,21
2261	174,5	55,84	437	82	7,34
2275	81,5	11,54	462	128,5	16,8
2280	116,5	12,12	486	147	16,6
2281	88,2	17,37	493	78,5	18,06
2282	75,5	15,1	494	101	10,1
2289	162	8,67	496	143	15,03
2294	113	10,21	507	117,5	13,6
2311	137	24,4	518	152,5	11,5
2312	129,5	12,12	629	107,5	20,4
2316	162,5	10,67			
562	157	14,37	WT	100	3,2

FIGURE 19

409/462	409/2289	507/2312	2289/629
409/507	409/2316	507/2289	2312/2289
409/629	409/2294	507/2316	2312/2316
462/507	462/2312	507/2294	2312/2294
462/629	462/2289	2312/629	2289/2316
507/629	462/2316	2316/629	2289/2294
409/2312	462/2294	2294/629	2316/2294

FIGURE 20

	TD antibody Abolition to inhibition (%)	GC antibody Abolition to inhibition (%)	SL antibody Abolition to inhibition (%)	PR antibody Abolition to inhibition (%)	Specific activity (mUOD/ng/ml)
409/462	49	93	0	19	19,7
409/507	57	71	28	51	9,4
409/629	19	ND	ND	ND	6,14
462/507	57	57	6	49	12,1
462/629	33	93	94	43	7,1
507/629	12	12	0	10	7,04

FIGURE 21

VIII FACTORS FOR THE TREATMENT OF TYPE A HEMOPHILIA

CROSS-REFERENCE TO RELATED APPLICATION

This application is the U.S. national stage application of International Patent Application No. PCT/FR2008/050301, filed Feb. 22, 2008, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

FIELD OF THE INVENTION

The present invention relates to the field of hemostasis, more specifically to that of hemophilia A. The invention relates to human factor VIII variants and to the uses thereof.

TECHNICAL BACKGROUND

Factor VIII (FVIII) is mainly synthesized by hepatocytes and sinusoidal endothelial cells. The plasma concentration of FVIII is comprised between 0.1 and 0.2 mg/l; the circulating form is inactive and associates with von Willebrand factor (vWF). FVIII plays a key role in the endogenous (so-called intrinsic) pathway of blood coagulation. When a blood vessel is damaged by trauma, bleeding is triggered. In response, the process of hemostasis is initiated, consisting of a complex chain of events leading to the formation of a blood clot which seals the site of injury. Blood coagulation begins when platelets adhere to injured vessel walls. If the injury is severe, the platelet aggregates at the site of injury are insufficient to form a hemostatic plug to staunch the blood flow. Thus coagulation factors intervene whose purpose is to form the fibrin network, generated from soluble fibrinogen molecules by the action of thrombin. The formation of this network composed of insoluble fibers is crucial to firmly anchor the blood clot. Cascade shall be understood to mean that, sequentially and at each step, a precursor protein is converted to an activated protease which cleaves or acts as cofactor for cleavage of the next precursor protein of the cascade. Thus, FVIII is proteolytically cleaved in FVIIIa by the action of thrombin and factor Xa. In this active procoagulant form (FVIIIa), FVIII strikingly increases the proteolytic efficiency of factor FIXa towards factor FX.

Hemophilia A is a bleeding disorder characterized by a deficiency of activated FVIII due to a mutation in the recessive gene encoding FVIII. In some rare cases, hemophilia A may arise from the spontaneous development of auto-antibodies directed against FVIII; this is known as acquired hemophilia A.

Hemophilia is manifested as a defect of blood clotting in response to a hemorrhage. Untreated type A hemophiliacs exhibit symptoms such as excessive bleeding after trauma and sometimes even spontaneous hemorrhages, particularly into the articulation joints. Hemophilia A is the most common coagulation disorders and occurs in 1 in 5,000-10,000 male births. Not all hemophiliacs are affected in the same manner or to the same extent. For instance, hemophilia A is considered i) severe when FVIII levels are less than or equal to 1% of "normal" circulating levels; ii) moderate when FVIII levels are within the range of 1 to 5% of "normal"; and iii) mild when FVIII levels are between 5 and 30% of normal. These three types of hemophilia A occur at the following frequencies: 50% of hemophiliac patients have the severe form, 10% the moderate form and 40% the mild form.

Many genetic abnormalities have been associated with the gene coding for FVIII. Said gene is located at the tip of the long arm of the X chromosome (locus Xq28). Hemophilia A results from an abnormality in this gene. It is an X-linked recessive disorder: males and females can transmit the disorder but only males are affected. The molecular defects may be gene mutations, deletions or inversions. The majority of patients harboring missense point mutations have mild or moderate disease. Deletions are classified into two types: i) small deletions; ii) large deletions (>1 kb). Most large deletions confer a severe phenotype. With respect to genetic inversions, the intron 22 inversion is the most frequent and is responsible for the majority of cases of severe hemophilia A (45%). Another inversion, the intron 1 one, can cause severe disease while less frequent (3%).

In summary, these mutations result in either a decreased production of functionally normal FVIII molecules, or a quantitatively normal production of functionally defective FVIII molecules.

The FVIII gene codes for a polypeptide chain of 2,351 amino acids (aa) (SEQ ID No. 2) corresponding to a 19 aa signal peptide and a 2332 aa mature protein (330 kDa) (SEQ ID No. 3). The nucleotide sequence of the FVIII precursor is given in SEQ ID No. 1 and the corresponding protein sequence in SEQ ID No. 2. The FVIII precursor consists of a succession of the following seven functional domains: A1, a1, A2, a2, B, a3, A3, C1 and C2, from the N-terminal to the C-terminal (Vehar et al., 1984, Nature, 312:337-342).

FVIII undergoes a first intracellular proteolysis at arginines 1313 and 1648, producing a FVIII heterodimer consisting of: i) an A1-a1-A2-a2-B heavy chain; ii) an a3-A3-C1-C2 light chain. It circulates in plasma as a heterodimer. The interaction between the two chains is ensured among others by the presence of a chelated copper molecule in domains A1 and A3. Immediately after being secreted in plasma, FVIII forms a very high affinity association with von Willebrand factor (vWF) which protects it from proteases. FVIII and vWF form a noncovalent complex in which binding takes place mainly via two regions of FVIII: the N-terminal region and the C-terminal region at 2303-2332 (C2 domain) of the light chain. During coagulation, FVIII is cleaved by thrombin and factor Xa at three sites: i) thrombin cleaves at Arginine 1689 of the light chain and at Arginine 372 and Arginine 740 of the heavy chain; ii) factor XA cleaves FVIII at Arginine 336, Arginine 372 and Arginine 740. Two of these cleavages are common (Arginine 372 and Arginine 740). Cleavages at Arginine 372 and Arginine 1689 are essential for FVIII to participate in the coagulation cascade. These cleavages activate FVIII, also known as FVIIIa ("a" for "active"); in addition to FVIIIa activation, these cleavages result in removal of the 170 kDa B domain and dissociation of FVIIIa from vWF.

The B domain of FVIII, defined by amino acids 741 to 1648, can be totally or partially deleted with no loss of activity of recombinant FVIII (Toole et al., 1986, Proc. Natl. Acad. Sci. USA, 83 (16):5939-5942; Eaton et al., 1986, Biochemistry, 25 (26):8343-8347; Langer et al., 1988, Behring Inst. Mitt, 82:16-25; Meulien et al., 1988, Protein Eng, 2(4):301-6; and U.S. Pat. No. 4,868,112), including for porcine FVIII (U.S. Pat. No. 6,458,563; WO01/68109; U.S. Pat. No. 6,770,744), which in some cases can be used to replace the human FVIII.

Mutations, most of them point mutations, can be inserted at different sites of FVIII without causing a loss of FVIII procoagulant activity (U.S. Pat. Nos. 5,744,446; 5,859,204; 6,060,447; 6,180,371; 6,228,620; 6,376,463; EP 1561757;

WO02/24723; WO97/49725). EP1502921 and WO2005/111074 describe human FVIIII variants with improved stability.

Other patents (US 2003/0083257; WO2005/040213; and U.S. Pat. No. 6,780,614) may be cited which describe modifications of FVIII cDNA for increasing its production in animal cells. The modifications of the cDNA are disclosed in patents US20021165177; US2002/0182684; EP1048726; EP1283263.

The number of units of FVIII administered is expressed in International Units (IU) with reference to the WHO standard for FVIII. FVIII activity is expressed either as a percentage (relative to normal human plasma) or in International Units (relative to an international standard). One International Unit (IU) of FVIII activity is equivalent to that quantity of FVIII contained in one milliliter of normal human plasma. Plasmatic FVIII assays may be carried out either by a chronometric method or by a chromogenic method.

Hemophilia A (severe and moderate forms) is generally treated by preventive or curative replacement therapy, which is based on repeated injections of the deficient coagulation factor or perfusion thereof. Patients with hemophilia A are treated with different types of plasma-derived or recombinant FVIII: i) recombinant; ii) semipurified plasma products; iii) plasma products purified on conventional or immunoaffinity columns. The first recombinant FVIII concentrates contained albumin as stabilizing agent. These included Kogenate® (Bayer), Helixate® (manufactured by Bayer, distributed by Aventis), and Recombinate® (Baxter). New albumin-free formulations have been developed, such as Kogenate® FS (Bayer), Helixate® FS (Bayer), and ReFacto^{MC} (Wyeth). These nonetheless contain trace amounts of albumin arising from the cell culture medium used during the step of production of these recombinant proteins.

Recombinant human FVIII still needs to be optimized. Indeed, FVIII is relatively unstable in physiologic conditions, has a low activity in blood, is present at very low concentrations (0.1 to 0.2 μg/ml), and has a half-life of 10 to 12 hours.

In about 30% of severe hemophiliac A patients, replacement therapy causes complications specific to FVIII which lead to failure of the treatments usually used. In fact, after replacement therapy, patients may develop antibodies directed against the exogenous recombinant FVIII. These anti-FVIII antibodies inhibit the procoagulant activity of FVIII, hence the name "inhibitory antibodies" or else "inhibitors". Further FVIII perfusion are rendered ineffective by these antibodies, and result in an increase of inhibitory antibody amount through a phenomenon known as "anamnestic reaction".

Rapidly, patients can no longer be treated with FVIII, in which case the inhibitor "titer" is determined. This titer is expressed in international Bethesda units (BU). One BU of inhibitors corresponds to inactivation of half of the amount of FVIII in 1 ml of normal plasma. A titer is "low" when less than 10 BU, and "high" when more than 10 BU.

When the inhibitor titer is relatively low, hemophiliac patients may be given the aforementioned FVIII concentrates such as Kogenate® FS, Helixate® FS, Recombinate®, and ReFacto^{MC}, but this carries a significant risk of inducing a rise in inhibitor titers which must therefore be closely monitored.

One of the ways to control inhibitory antibodies is to induce immune tolerance through administration of large doses of FVIII according to "de Bonn" protocol. In some patients, the inhibitory antibody titer is so high that they cannot be treated with large doses of FVIII for toxicity reasons.

A second approach known as the "Bonn-Malmo protocol" is based on one hand on ex vivo immunoadsorption of inhibitors immediately followed by reinjection of the blood, and on the other hand on injection of large doses of FVIII combined with immunosuppressive agents. These treatments are extremely costly in terms of recombinant FVIII and have achieved partial success.

Another approach consists in supplying coagulation factors in order to "bypass" the requirement of FVIII in the intrinsic coagulation pathway by using: i) plasma-derived activated prothrombin complex (FEIBA® VH, Factor Eight Inhibitor Bypassing Activity; Baxter) containing Factors II, VII, IX and X; ii) recombinant activated Factor VIIa (rFVIIa; NovoSeven®/Niastase®; NovoNordisk).

Said approaches have clear-cut success, nevertheless counterbalance by the development of side effects associated with this type of therapy (such as additional bleeding or conversely thrombotic events related to the frequency of administration).

It should be noted that circulating FVIII level increases after injection and then gradually declines related to its half-life. FVIII half-life ranges from 8 to 16 hours, with an average of 12 hours, raising the problem of repeated injections.

Another option consists in using a porcine FVIII with the aim to avoid antibodies directed against human FVIII. Patients who developed inhibitors to human FVIII have been successfully treated with semi-purified porcine FVIII (Hyate:C). Yet, this success has only been partial because after several injections of porcine FVIII, anti-porcine FVIII inhibitors have also developed, as mentioned in US2004/0249134. This phenomenon may necessitate to end treatment. Ipsen and Octagen are now co-developing a recombinant porcine FVIII known as OBI-1 in collaboration with Emory University in the USA, as a replacement for Hyate:C (WO2005107776).

Administration of porcine FVIII is therefore not a definitive solution for the treatment of hemophilia A patients with inhibitors.

As it can be seen, today there is no ideal treatment for individuals with hemophilia A, with or without inhibitors. The various problems encountered with commercial FVIII-based treatments associated with the development of these inhibitory antibodies have driven efforts to rapidly design a novel FVIII which has retained procoagulant specific activity and having lost the epitopes recognized by the inhibitory antibodies.

Few studies have addressed the epitope specificities of "inhibitory" antibodies. Some inhibitory antibodies appear to recognize small regions of the FVIII molecule: i) C2 domain in the light chain (2181-2321); ii) A2 domain in the heavy chain (484-509); iii) A3 domain (1694-2019) (Prescott et al., 1997, Blood, 89:3663-3671; Barrow et al., 2000, Blood, 95:557-561).

The 18 kDa C2 domain, between Serine 2173 and Tyrosine 2332, contains the membrane phospholipid binding domain and a part of the vWF binding domain. Inhibitory antibodies directed against the C2 domain mainly block the binding to phospholipids binding required for procoagulant activity but also the interaction with vWF. Mutations at positions Methionine 2199, Phenylalanine 2200, Valine 2223, Lysine 2227, Leucine 2251 and Leucine 2252 illustrate the importance of these amino acids in FVIIII activity and binding to phospholipids and/or to vWF (Pratt et al., 1999, Nature, 402:439-442).

Anti-A2 antibodies inhibit the function of FVIIIa as cofactor of Factor X (Lollar et al., 1994, J. Clin. Invest. 93:2497-2504). The main A2 epitope has been located between Arginine 484 and Leucine 508 (Healey et al., 1995, J. Biol. Chem., 270:14505-14509).

Antibodies directed against A3 and/or C2 domain prevent stabilization of the interaction between FVIII and vWF and also interfere with binding of the FVIII light chain to activated FIX.

Inhibitors are very heterogeneous from one patient to another and epitope specificity may change over time. Kinetic study of FVIII inhibition have revealed two types of allo-antibodies: type I antibodies which completely neutralize exogenous FVIII, and type II antibodies which never totally inhibit FVIII activity. Type II antibodies not completely block the procoagulant activity of FVIII because they are not saturable or display decreasing affinity according to their concentration.

Regions which can be recognized by inhibitory antibodies are cited in patents US2003/147900 and WO00/48635. These exposed and antigenic FVIII regions are between positions 1649-2019, 108-355, 403-725 and 2085-2249.

Moreover, US 2005/0256304 describes the following set of positions in human FVIII, where substitutions are likely to decrease antigenicity: 197, 198, 199, 201, 202, 407, 411, 412, 419, 515, 517, 613, 617, 636, 637, 638, 639, 823, 1011, 1013, 1208, 1209, 1210, 1254, 1255, 1257, 1262, 1264, 1268, 1119, 1120, 1121, 1122, and 1123.

The antigenicity of human FVIII can be decreased by glycosylation of recognition sites of inhibitors. Said method is disclosed in U.S. Pat. No. 6,759,216 and JP2004141173.

Another option consists in substituting the human FVIII epitopes usually recognized by inhibitors in domains: i) A2 (484-509); ii) A3 (1694-2019), a3 (1649-1687); iii) C2 (2181-2321). This solution is based on the use of a hybrid recombinant protein: a human/porcine FVIII.

The main targets of inhibitory antibodies are located in the A2 and C2 domains of FVIII (Saenko et al., Haemophilia, 2002). In fact, it is generally thought that 90% of inhibitory antibodies are directed against the human A2 and C2 domains (Barrow et al., 2000, Blood, 95:564-569). Moreover, it has been shown that human inhibitors have weak activity against porcine FVIII (Koshihara et al., Blood, 1995).

It is therefore expected that a substitution of human FVIII epitopes by porcine sequences would lead to a hybrid molecule less reactive towards inhibitory antibodies. Thus, the human A2 and C2 domains were replaced by their corresponding porcine domains (Lubin et al., 1994, J. Biol. Chem., 269:8639-8641). However, once again, anti-porcine FVIII antibodies eventually developed during the treatment of patients with inhibitors.

Many patents describe human/animal FVIII hybrids having retained a procoagulant activity. Human/animal hybrid, as used herein, denotes any combination (substitution) of at least one amino acid between a human FVIII sequence and a FVIII sequence of animal origin. Said hybrids have been produced, on the one hand, by substituting regions (functional subunits or structural domains) by the corresponding animal regions. For instance, U.S. Pat. Nos. 5,888,974; 5,663,060; 5,583,209; EP1359222; U.S. Pat. No. 5,744,446; WO93/20093; and WO95/24427 provide hybrid FVIII molecules derived from combinations of heavy and light chains of human and non-human FVIII, and/or derived from combinations of human/porcine FVIII domains.

U.S. Pat. No. 5,744,446 describes human/porcine FVIII variants wherein sequences of the human A2 domain are substituted by the corresponding murine or porcine sequences. The substituted fragments of the A2 domain are: 373-540; 373-508, 445-508, 484-508, 404-508, 489-508 and 484-489.

U.S. Pat. No. 5,364,771 provides a method for purifying FVIII hybrids derived from combinations of light and heavy chains from human and non-human FVIII: human FVIII in which the A2 domain is replaced by the porcine A2 domain.

On the other hand, in some patents, said hybrids are formed by point substitutions of one or several amino acids of human FVIII by the corresponding amino acid(s) of animal origin (porcine, canine or murine). For example, US2004/0197875 discloses modifications in codon charges at certain positions of human FVIII. Said positions are defined related to porcine FVIII sequence. EP1454916 describes the introduction of porcine codons into the human cDNA.

Among these patents, studies have been addressed to develop human/porcine FVIII hybrids in the region of the A2 domain. EP1359222 describes a study of the porcine A2 domain sequence, with a view to generating such hybrid. US2003/166536; U.S. Pat. No. 6,376,463; WO00/71141 describe amino acid substitutions in human FVIII at key epitopes in the A2 domain, between positions 484 and 508: 486, 490, 491, 493, 494, 496, 498, 499, 500, 502, 503, 504, 505, 506, 507 for WO00/71141; and 485, 487, 488, 489, 492, 495, 501, 508 for U.S. Pat. No. 5,859,204. In particular, Alanine substitutions were made at positions: Arginine 484, Proline 485, Tyrosine 487, Serine 488, Arginine 489, Proline 492, Valine 495, Phenylalanine 501, and Isoleucine 508. These substitutions conferring decreased antigenicity might be of interest from a therapeutic standpoint.

Likewise, in U.S. Pat. No. 6,180,371, Arginine 484 is substituted by Serine, Proline 485 by Alanine, Arginine 489 by Glycine, Proline 492 by Leucine. With these variants, inhibition of the procoagulant function of FVIII by antibodies was alleviated or disappeared altogether. The therapeutic interest of a double or triple mutant at Arginine 484, Arginine 489 and Phenylalanine 501, where each codon is substituted with an Alanine, is suggested.

There are also patents disclosing FVIII variants in which the substitutions only affect the C2 domain.

US2004/249134; WO03/047507; WO02/24723; U.S. Pat. No. 6,770,744 describe substitutions at positions Methionine 2199, Phenylalanine 2200, Valine 2223, Lysine 2227, Leucine 2251 and Leucine 2252. Said substitutions were introduced into a FVIII lacking the B domain. Amino acids at positions 2215, 2220, 2320, 2195, 2196, 2290 and 2313 were substituted with an Alanine.

With regard to position 2223, Valine is replaced by an Alanine, by comparison between human and porcine FVIII. This mutation is mentioned in Pratt's article "Structure of the C2 domain of human FVIII" (Nature, 1999, 402:439-442) and in U.S. Pat. No. 6,770,744.

Combinations of certain mutated positions such as 2199, 2200, 2223 and 2227 have been described as reducing the antigenicity of FVIII with regard to some anti-C2 domain inhibitory antibodies, all while retaining the coagulant activity of FVIII.

In patents WO99/46274 and US2005/0079584, J. Lollar's group describes a region of potential interest for constituting a less immunogenic FVIII: 2181 to 2243. This region was defined very roughly by an antigenicity study of human/porcine hybrids. An alignment between human and porcine FVIII of the sequence 2181 to 2243 disclosed 17 differences at the following positions: 2181, 2182, 2195, 2196, 2197, 2199, 2207, 2216, 2222, 2224, 2225, 2226, 2227, 2228, 2234, 2238, 2243. J. Lollar's group speculate that a substitution at these 17 positions by an Alanine, a Methionine, a Serine, a Glycine, or else a Leucine might generate a FVIII protein that can avoid inhibitory antibodies. This hypothesis is not supported by any antigenicity studies of mutants of interest.

Lastly, patents such as U.S. Pat. No. 6,180,371; US2002/182670; US 2003/068785; US2005/079584; WO99/46274;

U.S. Pat. No. 7,012,132; WO2005/046583 provide human/porcine hybrids harboring substitutions in both the A2 and C2 domains of FVIII with the aim of reducing inhibition by inhibitory antibodies that recognize both domains. In particular, WO2005/046583 describes amino acid substitutions in the A2 and C2 domains at positions 484, 489, 492, 2199, 2200, 2251 and 2252. The FVIII which was used lacks the B domain. Only position 484 has an Arginine substituted by an Alanine.

To summarize, while many studies make reference to novel FVIII variants, there is still a need for a novel, less immunogenic FVIII, because there are no modified FVIII variants capable of treating patients with inhibitors currently on the market. Moreover, variants with an improved specific activity or an improved capacity to be secreted are also of major interest to promote the production of recombinant FVIII or to improve the treatment of patients.

SUMMARY OF THE INVENTION

The present invention therefore provides novel improved FVIII variants. Said variants may have lost the epitopes recognized by inhibitory antibodies all while retaining the core of their procoagulant activity, or have an improved specific activity, or else have an improved secretion capacity. Said variants may also have a combination of these features. For example, the invention relates to variants which are less immunogenic and have an improved specific activity and/or an improved secretion capacity. Likewise, the invention relates to variants having an improved specific activity and/or an improved secretion capacity.

A first object of the present invention is an improved human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507, 629, 400, 562, 403, 518, 414, 496, 421, 493, 486, and 494 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain. In a particular embodiment, the human FVIII variant or biologically active derivative thereof consists of a single substitution. In another particular embodiment, the human FVIII variant or biologically active derivative thereof further comprises a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. In a particular embodiment, the human FVIII variant or biologically active derivative thereof comprises the substitution of at least two, three, four, five six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen amino acids, preferably selected from the aforementioned groups. Preferably, the amino acid is substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine. More preferably, the amino acid is substituted by an Alanine. Preferably, the biologically active FVIII derivative is a FVIII consisting in a partial or whole deletion of the B domain.

In a particular embodiment, the variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII and retains a procoagulant activity at least equal to 50% of that of natural human FVIII. In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312, and 2316 of the C2 domain. Said variant can further comprise a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. In a more preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507 and 629 of the A2 domain. In another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising or consisting of the combination of two substitutions selected from the group consisting of 409+462, 409+507, 462+507, 409+629, 462+629, 507+629. In yet another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising or consisting of the combination of three substitutions selected from the group consisting of 409+462+507, 462+507+629, 409+462+629, 409+507+629. In another particular embodiment, the invention relates to an improved human FVIII variant or biologically active derivative thereof comprising or consisting of the combination of four substitutions at positions 409, 462, 507 and 629.

Furthermore, these mutations which confer abolition to inhibition by inhibitory antibodies may prove to be of great interest in combination with mutations conferring a higher specific activity, allowing compensating an optional relative loss of activity of these less antigenic mutants. In a particular embodiment, the variant has an improved specific activity as compared to that of natural human FVIII. In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof further comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain.

Said mutations which confer abolition to inhibition by inhibitory antibodies may also prove to be of great interest in combination with mutations conferring an improved capacity to be secreted, by allowing compensating an optional relative loss of secretion of these less antigenic mutants. In a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof further comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain. Massive production of mutants having retained at least 50% of FVIII activity also makes it possible to encompass their use in a context of analyzing additional functions of the protein. In addition to a modulation of its immunogenicity, secretion and specific activity, the following properties of FVIII might be improved by using the herein described mutated molecules: —binding to von Willebrand factor and therefore improved half-life of FVIII or circulating FVIIIa; —improved intrinsic stability of the molecule by stabilization of the A2 domain and therefore an increased efficiency period; —binding to phospholipids derived from blood platelets, cell surfaces or circulating microparticles and therefore improved formation of FXa; —binding to FIXa and FX and therefore improved formation of FXa; —decreased binding of FVIII or FVIIIa to the molecules responsible for its catabolism such as for example low density Lipoprotein Receptor-related Protein (LRP), Low density Lipoprotein Receptor (LDLR), Very Low Density Lipoprotein Receptor (VLDLR), megaline or any other receptor which might be identified and therefore improved half-life of circulating FVIII; —proteolysis decrease of FVIII or FVIIIa by vascular proteases such as for example activated protein C, FXa, FIXa, and therefore increase efficiency period.

A second object of the present invention relates to a nucleic acid coding for a human FVIII variant or a biologically active derivative thereof according to the invention, an expression cassette comprising said nucleic acid, a vector, preferably an expression vector, comprising said nucleic acid or said expression cassette, and a host cell comprising a nucleic acid, an expression cassette or a vector according to the present invention. Preferably, the vector can be selected from a plasmid and a viral vector. The present invention also relates to the use of a nucleic acid, an expression cassette, an expression vector or a host cell according to the invention for producing a human FVIII variant or a biologically active derivative thereof according to the present invention.

A third object of the present invention relates to a pharmaceutical composition comprising a human FVIII variant or a biologically active derivative thereof according to the invention. Thus, the present invention relates to a human FVIII variant or a biologically active derivative thereof according to the invention as medicament. The present invention further relates to a human FVIII variant or a biologically active derivative thereof according to the invention for the treatment of hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. In another embodiment, the patient to be treated is a hemophiliac patient before any development of inhibitors. The present invention equally relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the present invention.

A fourth object of the present invention relates to the use of a human FVIII variant or a biologically active derivative thereof according to the invention for preparing a medicament for the treatment of hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. In another embodiment, the patient to be treated is a hemophiliac patient before development of any optional inhibitors. The present invention also relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the present invention.

A fifth object of the present invention relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the present invention for the diagnosis of inhibitor type in a patient with hemophilia A.

BRIEF DESCRIPTION OF FIGURES AND TABLES

FIG. **1**: Simplified scheme of the coagulation cascade. Ca: calcium-dependent step. PL: phospholipids of blood platelet membrane. TF: tissue factor. TFPI: tissue factor pathway inhibitor. The role of FVIIIa is to increase the catalytic efficiency of FIXa to activate FX. Assembly of FXa and FVa triggers a significant increase in thrombin formation.

FIG. **2**A-**2**E: Primary screen results: Raw activities of 359 Alanine mutants over the 795 produced=functional mapping of FVIII activity of these 359 positions.

FIG. **3**: Production of FVIII in culture medium; 8 mutants displayed a much higher production level than non-mutated FVIII in the same conditions.

FIG. **4**: Highest specific activities of 15 mutants compared to non-mutated FVIII in the same conditions.

FIG. **5**: Example of determining abolition of the serum TD to inhibition by FVIII mutant E518A. Abolition to inhibition is expressed as a percentage: $[(b-a)/a]\times100$; where "a" represents residual activity percentage of the WT (serum+IgG/serum−IgG) and "b" is the residual activity percentage of the mutant (serum+IgG/serum−IgG).

FIG. **6**A-**6**E: Abolition of FVIII-4A2 versus wild-type FVIII to inhibition by inhibitory antibodies from five patients (TD, GC, PR, SL and FS) measured by Bethesda assay.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody; the residual activity percentage is thus determined.

FIG. **7**A-**7**B: Determining the inhibition decrease of the FVIII-4A2 mutant by anti-A2 domain antibody (GMA012) and a rabbit polyclonal antibody.

FIG. **8**A-**8**B: Comparative titration on a solid support of FVIII-4A2 versus wild-type FVIII by ELISA using anti-C2 domain antibody (ESH4) and anti-A2 domain antibody (GMA012).

FIG. **9**: Comparative determination of FVIII-4A2 and wild-type FVIII activation by thrombin.

FIG. **10**: Comparative determination of A2 domain dissociation and resultant loss of activity for FVIII-4A2 and wild-type FVIII after activation by thrombin (IIa).

FIG. **11**A-**11**D: Abolition of FVIII-3A2 versus wild-type FVIII to inhibition by inhibitory antibodies from four patients (TD, GC, SL and FS) measured by Bethesda assay.

FIGS. **12**-**14**: Primary screen results; list of **158** Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

FIGS. **15**-**18**: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. **5**.

FIG. **19**: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the **30** mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

FIG. **20**: List of all FVIII double mutants produced from the eight single mutants FVIII**409**A, FVII**1462**A, FVIII**507**A, FVIII**629**A, FVIII**2289**A, FVIII**2294**A, FVIII**2312**A and FVIII**2316**A.

FIG. **21**: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A**2** mutants from sera of four hemophiliac patients TD, GC, SL and PR.

Description of the invention

The present invention provides a solution to resolve a serious complication that occurs in **30**% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an optional relative loss of activity in variants whose mutations confer a abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate a optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: **5409**A indicates the substitution of the serine residue at position **409** of SEQ ID No. **3** by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non- naturally occuring amino acid. The terms "substitution" and "mutation" are interchangeable. The sign "+" indicates a combination of substitutions.

"Comprise" means that the variant or the fragment thereof has one or more substitutions such as indicated with reference to SEQ ID No. **3**, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the **158** selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier's instructions. Briefly, culture supernatants (50 μl) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50 μ**1**), preheated at 37° C., was then added for 4 min, after which 50 μl of development medium at 37° C. were added. The formation of product over time was measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

FIGS. **12**-**14** show the activities of the **158** mutants which retained more than 50% of non-mutated FVIII activity. Said **158** mutants were selected for the secondary screening.

Example **4**: Secondary screen: Evaluation of loss of antigenicity towards human FVIII inhibitory antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the **158** mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chronometric assay.

Culture supernatants obtained after **48** h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chronometric activity comprised in the range of about 10-20% (1 FVIII unit =100% activity =200 ng/ml). The culture supernatant diluted or not (140 μl) was added to 150 μl of FVIII-depleted human plasma (Stago, Asnieres, France). An antibody dilution (10 μl) was then added to the mix. These antibodies are IgG fractions purified on protein A- from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained. Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1h**30**. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-l'Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient's serum. Said percentage was calculated as shown in FIG. **5** for the FVIII mutant E**518**A. Abolition to inhibition expressed is a percentage =−[(b−a)/a] x 100;where "a" is the percentage residual activity of the WT (serum + IgG / serum—IgG) and "b" is the percentage residual activity of the mutant (serum + IgG/serum −IgG).

FIGS. **15**-**18** show for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant **2316** for sera TD and SL, mutant **2294** for serum GC, mutant **403** for serum FS and mutant **2275** for serum PR.

Patients' sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIIIs produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least **50**% of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). FIG. **19** shows comparative data of raw and specific activities of **30** mutants selected in the secondary screen.

The eight FVIII Alanine mutants **2175**, **2199**, **2200**, **2215**, **2251**, **2252**, **2278** and **2316** displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. **3** depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants **2177**, **2183**, **2186**, **2191**, **2196**, **2204**, **2205**, **2206**, **2213**, **2217**, **2235**, **2258**, **2264**, **2268** and **2269** displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15mutants are given in FIG. **4**. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

Example **5**: Selection and combination of the best single mutants selected in the secondary screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophiliac patients with inhibitors;
- raw coagulant activity at least **100**% relative to non-mutated FVIII; and
- reproducibly good level of expression.

The eight selected mutants were mutants **409**, **462**, **507** and **629** in the A**2** domain and mutants **2289**, **2294**, **2312** and **2316** in the C**2** domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in FIG. **19**. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations **409**, **462**, **507**, **629**, **2289**, **2294**, **2312** and **2316** (six A**2** double mutants +six C**2** double mutants +sixteen A**2**-C**2** double mutants presented in FIG. **20**) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-**7** mammalian cells as described in Example **2**. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophiliac patients. The A**2** double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C**2** domain mutations (six C**2** double mutants +sixteen A**2**-C**2** double mutants) displayed an insignificant or null abolition to inhibition.

FIG. **21** shows the specific activities of the six A**2** double mutants and their percentage of abolition to inhibition by sera from four hemophiliac patients TD, GC, SL and PR calculated as in Example **4**. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A**2** domain. The choice was therefore based on the combination of the four mutations **409**, **507**, **462** and **629**. Triple mutants and the quadruple mutant comprising these four mutations **409**, **507**, **462** and **629** were also constructed.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

Table 1: Primary screen results; list of 158 Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

Table 2: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. **5**.

Table 3: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

Table 4: List of all FVIII double mutants produced from the eight single mutants FVIII409A, FVIII462A, FVIII507A, FVIII629A, FVIII2289A, FVIII2294A, FVIII2312A and FVIII2316A.

Table 5: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

DESCRIPTION OF THE INVENTION

The present invention provides a solution to resolve a serious complication that occurs in 30% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an optional relative loss of activity in variants whose mutations confer an abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may be interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: S409A indicates the substitution of the serine residue at position 409 of SEQ ID No. 3 by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non-naturally occurring amino acid. The terms "substitution" and "mutation" are interchangeable. The sign "+" indicates a combination of substitutions.

"Comprise" means that the variant or the fragment thereof has one or more substitutions such as indicated with reference to SEQ ID No. 3, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

"Consists of" means that the variant or the fragment thereof contains only the substitution(s) indicated with reference to SEQ ID No. 3.

"Variant" refers in particular to a polypeptide which differs from a polypeptide represented by sequence SEQ ID No. 3 by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 residue(s), preferably by 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 residues.

Amino acids of the A2, A3 or C2 domains of FVIII were systematically substituted by an Alanine. The production of these human FVIII mutants was carried out in mammalian cells. The primary screening of these variants was based on their procoagulant activity. The raw activity of each mutant was measured by chromogenic assay and compared with chromogenic assay of non-mutated human FVIII as reference. The activity of the FVIII variants can be determined by any method known to one skilled in the art, preferably according to method described in example 3 herein after. The FVIII variants selected as being the most active in the primary screen were then assessed for a second feature: loss of antigenicity towards sera from hemophiliac patients selected for their capacity to inhibit FVIII activity. Said secondary screening with said antibodies corresponds to a modified Bethesda assay. The antigenicity modification of the FVIII variants can be determined by any method known to one skilled in the art, preferably according to the method described in example 4 below.

Improved variants could be selected. Not only did some of these candidate medicaments retain a coagulant activity, but they also partially avoided inhibition by inhibitory antibodies from the sera of selected hemophiliac patients. These FVIIIs have lost one or more epitopes usually recognized by inhibitory antibodies from patients' sera. Furthermore, the candidate medicaments had a specific coagulant activity higher to that of wild-type FVIII. Another interesting feature is that the candidate medicaments displayed an improved secretion capacity.

In one embodiment, the invention therefore relates to recombinant human FVIII variants having lost at least one of the epitopes usually recognized by anti-FVIII antibodies known as "inhibitors", while retaining a coagulant activity, preferably higher, similar or close to that of non-mutated FVIII.

The present invention describes human FVIII variants comprising at least one substitution of an amino acid by an Alanine or any other amino acid in the C2 and A2 domains.

In particular the invention describes 158 Alanine mutants of human FVIII. "Alanine mutant", as used herein, denotes a mutant comprising the substitution of an amino acid by an Alanine residue. In particular, said mutants have an Alanine substitution at a residue located among the positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324 of the C2 domain and the positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642 of the A2 domain.

The positions of the residues are indicated with reference to the protein sequence of the 2332 amino-acid human FVIII, as illustrated in SEQ ID No. 3.

The invention relates to a human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid of the C2 domain selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324. The variant can further comprise a substitution of at least one residue at position 2175, 2195, 2196, 2202, 2215 and 2222. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. Said amino acids, among the twenty naturally occurring amino acids, are known to decrease the antigenicity of a protein. The substitution or substitutions at these positions, in particular by an Alanine, result in an improved FVIII variant, in particular having lost one or more epitopes recognized by inhibitory antibodies and having retained its procoagulant activity. The present invention also relates to a FVIII light chain comprising a substitution of at least one amino acid of the C2 domain selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324. This light chain can further comprise a substitution of at least one residue at position 2175, 2195, 2196, 2202, 2215 and 2222.

The invention further relates to a human FVIII variant or a biologically active derivative thereof comprising or containing a substitution of at least one amino acid of the A2 domain, preferably selected from the group consisting of the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642. The variant can further comprise a substitution of at least one residue at position 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. The substitution or substitutions at these positions, in particular by an Alanine, result in an improved FVIII variant, in particular having lost one or more epitopes recognized by inhibitory antibodies and having retained its procoagulant activity. The present invention also relates to a FVIII heavy chain, optionally which totally or partially lacks the B domain, comprising a substitution of at least one amino acid of the A2 domain selected from the group consisting of the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642. The variant can further comprise a substitution of at least one residue at position 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623.

The invention further relates to a human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid comprising or containing a substitution of at least one amino acid selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324 of the C2 domain and the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642 of the A2 domain. In a particular embodiment, the human FVIII variant or the biologically active derivative thereof further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2195, 2196, 2202, 2215 and 2222 of the C2 domain and the residues at positions 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623 of the A2 domain. In a particular embodiment, the human FVIII variant or the biologically active derivative thereof comprises the substitution of at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen amino acids, preferably selected from the aforementioned groups.

In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain and the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain. In another embodiment, said variant can further comprise a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. In a particular embodiment, said human FVIII variant or biologically active derivative thereof has a single substitution. Said single substitution is preferably selected from the group consisting of the substitutions L400A, L400M, L400S, L400G, D403A, D403M, D403S, D403G, D403L, S409A, S409M, S409G, S409L, N414A, N414M, N414S, N414G, N414L, R421A, R421M, R421S, R421G, R421L, L462A, L462M, L462S, L462G, L486A, L486M, L486G, K493M, K493S, K493G, K493L, G494A, G494M, G494L, K496A, K496S, K496G, K496L, E507A, E507M, E507S, E507L, E518A, E518M, E518S, E518G, E518L, R562A, R562M, R562S, R562G, R562L, V629A, V629M, V629S, V629G and V629L in the A2 domain and the substitutions S2206A, S2206G, S2206M, S2206L, L2212A, L2212M, L2212S, L2212G, P2226A, P2226M, P2226S, P2226G, P2226L, T2244A, T2244M, T2244S, T2244G, T2244L, L2261A, L2261M, L2261S, L2261G, F2275A, F2275M, F2275S, F2275G, F2275L, V2280A, V2280M, V2280S, V2280G, V2280L, K2281A, K2281M, K2281S, K2281G, K2281L, V2282A, V2282M, V2282S, V2282G, V2282L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, Q2311A, Q2311M, Q2311S, Q2311G, Q2311L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L in the C2 domain. In another embodiment, the invention relates to a human FVIII variant or a biologically active derivative thereof comprising at least one substitution selected from the group consisting of the substitutions L400A, L400M, L400S, L400G, D403A, D403M, D403S, D403G, D403L, S409A, S409M, S409G, S409L, N414A, N414M, N414S, N414G, N414L, R421A, R421M, R421S, R421G, R421L, L462A, L462M, L462S, L462G, L486A, L486M, L486G, K493M, K493S, K493G, K493L, G494A, G494M, G494L, K496A, K496S, K496G, K496L, E507A, E507M, E507S, E507L, E518A, E518M, E518S, E518G, E518L, R562A, R562M, R562S, R562G, R562L, V629A, V629M, V629S, V629G and V629L in the A2 domain and the substitutions S2206A, S2206G, S2206M, S2206L, L2212A, L2212M, L2212S, L2212G, P2226A, P2226M, P2226S, P2226G, P2226L, T2244A, T2244M, T2244S, T2244G, T2244L, L2261A, L2261M, L2261S, L2261G, F2275A, F2275M, F2275S, F2275G, F2275L, V2280A, V2280M, V2280S, V2280G, V2280L, K2281A, K2281M, K2281S, K2281G, K2281L, V2282A, V2282M, V2282S, V2282G, V2282L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, Q2311A, Q2311M, Q2311S, Q2311G, Q2311L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L in the C2 domain. Said FVIII variants have lost one or more epitopes usually recognized by said antibodies and therefore have decreased antigenicity as compared to non-mutated human FVIII. Furthermore, they have retained at least 50%, preferably at least 60 or 75%, of raw activity relative to non-mutated human FVIII.

In a still more preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and having retained at least 100% of raw activity as compared to non-mutated human FVIII, and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507, and 629 of the A2 domain and the residues at positions 2289, 2294, 2312, and 2316 of the C2 domain. In another embodiment, said variant can further comprise a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. In a particular embodiment, said human FVIII variant or biologically active derivative thereof has a single substitution. Said substitution is preferably selected from the group consisting of the substitutions S409A, S409M, S409G, S409L, L462A, L462M, L462S, L462G, E507A, E507M, E507S, E507L, V629A, V629M, V629S, V629G, V629L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L. In another embodiment, the invention relates to a human FVIII variant or a biologically active derivative thereof comprising at least one substitution selected from the group consisting of the substitutions S409A, S409M, S409G, S409L, L462A, L462M, L462S, L462G, E507A, E507M, E507S, E507L, V629A, V629M, V629S, V629G, V629L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L.

In a further embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and comprising the combination of two substitutions selected from the group consisting of 409+462, 409+507, 462+507, 409+629, 462+629 and 507+629, preferably 409+462, 409+507 and 462+507. In a particular embodiment, said human FVIII variant or biologically active derivative thereof comprises the combination of two substitutions selected from the group consisting of S409A+L462A, S409A+L462M, S409A+L462S, S409A+L462G, S409A+E507A, S409A+E507M, S409A+E507S, S409A+E507G, S409A+E507L, S409A+V629A, S409A+V629M, S409A+V629S, S409A+V629G, S409A+V629L, S409M+L462A, S409M+L462M, S409M+L462S, S409M+L462G, S409M+E507A, S409M+E507M, S409M+E507S, S409M+E507G, S409M+E507L, S409M+V629A, S409M+V629M, S409M+V629S, S409M+V629G, S409M+V629L, S409G+L462A, S409G+L462M, S409G+L462S, S409G+L462G, S409G+E507A, S409G+E507M, S409G+E507S, S409G+E507G, S409G+E507L, S409G+V629A, S409G+V629M, S409G+V629S, S409G+V629G, S409G+V629L, S409L+L462A, S409L+L462M, S409L+L462S, S409L+L462G, S409L+E507A, S409L+E507M, S409L+E507S, S409L+E507G, S409L+E507L, S409L+V629A, S409L+V629M, S409L+V629S, S409L+V629G, S409L+V629L, L462A+E507A, L462A+E507M, L462A+E507S, L462A+E507G, L462A+E507L, L462A+V629A, L462A+V629M, L462A+V629S, L462A+V629G, L462A+V629L, L462M+E507A, L462M+E507M, L462M+E507S, L462M+E507G, L462M+E507L, L462M+V629A, L462M+V629M, L462M+V629S, L462M+V629G, L462M+V629L, L462S+E507A, L462S+E507M, L462S+E507S, L462S+E507G, L462S+E507L, L462S+V629A, L462S+V629M, L462S+V629S, L462S+V629G, L462S+V629L, L462G+E507A, L462G+E507M, L462G+E507S, L462G+E507G, L462G+E507L, L462G+V629A, L462G+V629M, L462G+V629S, L462G+V629G, L462G+V629L, E507A+V629A, E507A+V629M, E507A+V629S, E507A+V629G, E507A+V629L, E507M+V629A, E507M+V629M, E507M+V629S, E507M+V629G, E507M+V629L, E507S+V629A, E507S+V629M, E507S+V629S, E507S+V629G, E507S+V629L, E507G+V629A, E507G+V629M, E507G+V629S, E507G+V629G, E507G+V629L, E507L+V629A, E507L+V629M, E507L+V629S, E507L+V629G and E507L+V629L, preferably in the group consisting of S409A+L462A, S409A+L462M, S409A+L462S, S409A+L462G, S409A+E507A, S409A+E507M, S409A+E507S, S409A+E507G, S409A+E507L, S409M+L462A, S409M+L462M, S409M+L462S, S409M+L462G, S409M+E507A, S409M+E507M, S409M+E507S, S409M+E507G, S409M+E507L, S409G+L462A, S409G+L462M, S409G+L462S, S409G+L462G, S409G+E507A, S409G+E507M, S409G+E507S, S409G+E507G, S409G+E507L, S409L+L462A, S409L+L462M, S409L+L462S, S409L+L462G, S409L+E507A, S409L+E507M, S409L+E507S, S409L+E507G, S409L+E507L, L462A+E507A, L462A+E507M, L462A+E507S, L462A+E507G, L462A+E507L, L462M+E507A, L462M+E507M, L462M+E507S, L462M+E507G, L462M+E507L, L462S+E507A, L462S+E507M, L462S+E507S, L462S+E507G, L462S+E507L, L462G+E507A, L462G+E507M, L462G+E507S, L462G+E507G and L462G+E507L.

In yet another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the combination of three substitutions selected from the group consisting of 409+462+507, 462+507+629, 409+462+629, 409+507+629, preferably 409+462+507. In a particular embodiment, said human FVIII variant or biologically active derivative thereof comprises the combination of three substitutions selected from the group consisting of S409A+L462A+E507A, S409A+L462A+E507M, S409A+L462A+E507S, S409A+L462A+E507G, S409A+L462A+E507L, S409A+L462M+E507A, S409A+L462M+E507M, S409A+L462M+E507S, S409A+L462M+E507G, S409A+L462M+E507L, S409A+L462S+E507A, S409A+L462S+E507M, S409A+L462S+E507S, S409A+L462S+E507G, S409A+L462S+E507L, S409A+L462G+E507A, S409A+L462G+E507M, S409A+L462G+E507S, S409A+L462G+E507G, S409A+L462G+E507L, S409M+L462A+E507A, S409M+L462A+E507M, S409M+L462A+E507S, S409M+L462A+E507G, S409M+L462A+E507L, S409M+L462M+E507A, S409M+L462M+E507M, S409M+L462M+E507S, S409M+L462M+E507G, S409M+L462M+E507L, S409M+L462S+E507A, S409M+L462S+E507M, S409M+L462S+E507S, S409M+L462S+E507G, S409M+L462S+E507L, S409M+L462G+E507A, S409M+L462G+E507M, S409M+L462G+E507S, S409M+L462G+E507G, S409M+L462G+E507L, S409G+L462A+E507A, S409G+L462A+E507M, S409G+L462A+E507S, S409G+L462A+E507G, S409G+L462A+E507L, S409G+L462M+E507A, S409G+L462M+E507M, S409G+L462M+E507S, S409G+L462M+E507G, S409G+L462M+E507L, S409G+L462S+E507A, S409G+L462S+E507M, S409G+L462S+E507S, S409G+L462S+E507G, S409G+L462S+E507L, S409G+L462G+E507A, S409G+L462G+E507M, S409G+L462G+E507S, S409G+L462G+E507G, S409G+L462G+E507L, S409L+L462A+E507A, S409L+L462A+E507M, S409L+L462A+E507S, S409L+L462A+E507G, S409L+L462A+E507L, S409L+L462M+E507A, S409L+L462M+E507M, S409L+L462M+E507S, S409L+L462M+E507G, S409L+L462M+E507L, S409L+L462S+E507A, S409L+L462S+E507M, S409L+L462S+E507S, S409L+L462S+E507G, S409L+L462S+E507L, S409L+L462G+E507A, S409L+L462G+E507M, S409L+L462G+E507S, S409L+L462G+E507G, S409L+L462G+E507L, S409A+L462A+V629A, S409A+L462A+V629M, S409A+L462A+V629S, S409A+L462A+V629G, S409A+L462A+V629L, S409A+L462M+V629A, S409A+L462M+V629M, S409A+L462M+V629S, S409A+L462M+V629G, S409A+L462M+V629L, S409A+L462S+V629A, S409A+L462S+V629M, S409A+L462S+V629S, S409A+L462S+V629G, S409A+L462S+V629L, S409A+L462G+V629A, S409A+L462G+V629M, S409A+L462G+V629S, S409A+L462G+V629G, S409A+L462G+V629L, S409M+L462A+V629A, S409M+L462A+V629M, S409M+L462A+V629S, S409M+L462A+V629G, S409M+L462A+V629L, S409M+L462M+V629A, S409M+L462M+V629M, S409M+L462M+V629S, S409M+L462M+V629G, S409M+L462M+V629L, S409M+L462S+V629A, S409M+L462S+V629M, S409M+L462S+V629S, S409M+L462S+V629G, S409M+L462S+V629L, S409M+L462G+V629A, S409M+L462G+V629M, S409M+L462G+V629S, S409M+L462G+V629G, S409M+L462G+V629L, S409G+L462A+V629A, S409G+L462A+V629M, S409G+L462A+V629S, S409G+L462A+V629G, S409G+L462A+V629L, S409G+L462M+V629A, S409G+L462M+V629M, S409G+L462M+V629S, S409G+L462M+V629G, S409G+L462M+V629L, S409G+L462S+V629A, S409G+L462S+V629M, S409G+L462S+V629S, S409G+L462S+V629G, S409G+L462S+V629L, S409G+L462G+V629A, S409G+L462G+V629M, S409G+L462G+V629S, S409G+L462G+V629G, S409G+L462G+V629L, S409L+L462A+V629A, S409L+L462A+V629M, S409L+L462A+V629S, S409L+L462A+V629G, S409L+L462A+V629L, S409L+L462M+V629A, S409L+L462M+V629M, S409L+L462M+V629S, S409L+L462M+V629G, S409L+L462M+

V629L, S409L+L462S+V629A, S409L+L462S+V629M, S409L+L462S+V629S, S409L+L462S+V629G, S409L+L462S+V629L, S409L+L462G+V629A, S409L+L462G+V629M, S409L+L462G+V629S, S409L+L462G+V629G, S409L+L462G+V629L, S409A+E507A+V629A, S409A+E507A+V629M, S409A+E507A+V629S, S409A+E507A+V629G, S409A+E507A+V629L, S409A+E507M+V629A, S409A+E507M+V629M, S409A+E507M+V629S, S409A+E507M+V629G, S409A+E507M+V629L, S409A+E507S+V629A, S409A+E507S+V629M, S409A+E507S+V629S, S409A+E507S+V629G, S409A+E507S+V629L, S409A+E507G+V629A, S409A+E507G+V629M, S409A+E507G+V629S, S409A+E507G+V629G, S409A+E507G+V629L, S409A+E507L+V629A, S409A+E507L+V629M, S409A+E507L+V629S, S409A+E507L+V629G, S409A+E507L+V629L, S409M+E507A+V629A, S409 M+E507A+V629M, S409M+E507A+V629S, S409M+E507A+V629G, S409M+E507A+V629L, S409M+E507M+V629A, S409M+E507M+V629M, S409M+E507M+V629S, S409M+E507M+V629G, S409M+E507M+V629L, S409M+E507S+V629A, S409M+E507S+V629M, S409M+E507S+V629S, S409M+E507S+V629G, S409M+E507S+V629L, S409M+E507G+V629A, S409M+E507G+V629M, S409M+E507G+V629S, S409M+E507G+V629G, S409M+E507G+V629L, S409M+E507L+V629A, S409M+E507L+V629M, S409M+E507L+V629S, S409M+E507L+V629G, S409M+E507L+V629L, S409G+E507A+V629A, S409G+E507A+V629M, S409G+E507A+V629S, S409G+E507A+V629G, S409G+E507A+V629L, S409G+E507M+V629A, S409G+E507M+V629M, S409G+E507M+V629S, S409G+E507M+V629G, S409G+E507M+V629L, S409G+E507S+V629A, S409G+E507S+V629M, S409G+E507S+V629S, S409G+E507S+V629G, S409G+E507S+V629L, S409G+E507G+V629A, S409G+E507G+V629M, S409G+E507G+V629S, S409G+E507G+V629G, S409G+E507G+V629L, S409G+E507L+V629A, S409G+E507L+V629M, S409G+E507L+V629S, S409G+E507L+V629G, S409G+E507L+V629L, S409L+E507A+V629A, S409L+E507A+V629M, S409L+E507A+V629S, S409L+E507A+V629G, S409L+E507A+V629L, S409L+E507M+V629A, S409L+E507M+V629M, S409L+E507M+V629S, S409L+E507M+V629G, S409L+E507M+V629L, S409L+E507S+V629A, S409L+E507S+V629M, S409L+E507S+V629S, S409L+E507S+V629G, S409L+E507S+V629L, S409L+E507G+V629A, S409L+E507G+V629M, S409L+E507G+V629S, S409L+E507G+V629G, S409L+E507G+V629L, S409L+E507L+V629A, S409L+E507L+V629M, S409L+E507L+V629S, S409L+E507L+V629G, S409L+E507L+V629L, L462A+E507A+V629A, L462A+E507A+V629M, L462A+E507A+V629S, L462A+E507A+V629G, L462A+E507A+V629L, L462A+E507M+V629A, L462A+E507M+V629M, L462A+E507M+V629S, L462A+E507M+V629G, L462A+E507M+V629L, L462A+E507S+V629A, L462A+E507S+V629M, L462A+E507S+V629S, L462A+E507S+V629G, L462A+E507S+V629L, L462A+E507G+V629A, L462A+E507G+V629M, L462A+E507G+V629S, L462A+E507G+V629G, L462A+E507G+V629L, L462A+E507L+V629A, L462A+E507L+V629M, L462A+E507L+V629S, L462A+E507L+V629G, L462A+E507L+V629L, L462M+E507A+V629A, L462M+E507A+V629M, L462M+E507A+V629S, L462M+E507A+V629G, L462M+E507A+V629L, L462M+E507M+V629A, L462M+E507M+V629M, L462M+E507M+V629S, L462M+E507M+V629G, L462M+E507M+V629L, L462M+E507S+V629A, L462M+E507S+V629M, L462M+E507S+V629S, L462M+E507S+V629G, L462M+E507S+V629L, L462M+E507G+V629A, L462M+E507G+V629M, L462M+E507G+V629S, L462M+E507G+V629G, L462M+E507G+V629L, L462M+E507L+V629A, L462M+E507L+V629M, L462M+E507L+V629S, L462M+E507L+V629G, L462M+E507L+V629L, L462S+E507A+V629A, L462S+E507A+V629M, L462S+E507A+V629S, L462S+E507A+V629G, L462S+E507A+V629L, L462S+E507M+V629A, L462S+E507M+V629M, L462S+E507M+V629S, L462S+E507M+V629G, L462S+E507M+V629L, L462S+E507S+V629A, L462S+E507S+V629M, L462S+E507S+V629S, L462S+E507S+V629G, L462S+E507S+V629L, L462S+E507G+V629A, L462S+E507G+V629M, L462S+E507G+V629S, L462S+E507G+V629G, L462S+E507G+V629L, L462S+E507L+V629A, L462S+E507L+V629M, L462S+E507L+V629S, L462S+E507L+V629G, L462S+E507L+V629L, L462G+E507A+V629A, L462G+E507A+V629M, L462G+E507A+V629S, L462G+E507A+V629G, L462G+E507A+V629L, L462G+E507M+V629A, L462G+E507M+V629M, L462G+E507M+V629S, L462G+E507M+V629G, L462G+E507M+V629L, L462G+E507S+V629A, L462G+E507S+V629M, L462G+E507S+V629S, L462G+E507S+V629G, L462G+E507S+V629L, L462G+E507G+V629A, L462G+E507G+V629M, L462G+E507G+V629S, L462G+E507G+V629G, L462G+E507G+V629L, L462G+E507L+V629A, L462G+E507L+V629M, L462G+E507L+V629S, L462G+E507L+V629G and L462G+E507L+V629L, preferably in the group consisting of S409A+L462A+E507A, S409A+L462A+E507M, S409A+L462A+E507S, S409A+L462A+E507G, S409A+L462A+E507L, S409A+L462M+E507A, S409A+L462M+E507M, S409A+L462M+E507S, S409A+L462M+E507G, S409A+L462M+E507L, S409A+L462S+E507A, S409A+L462S+E507M, S409A+L462S+E507S, S409A+L462S+E507G, S409A+L462S+E507L, S409A+L462G+E507A, S409A+L462G+E507M, S409A+L462G+E507S, S409A+L462G+E507G, S409A+L462G+E507L, S409M+L462A+E507A, S409M+L462A+E507M, S409M+L462A+E507S, S409M+L462A+E507G, S409M+L462A+E507L, S409M+L462M+E507A, S409M+L462M+E507M, S409M+L462M+E507S, S409M+L462M+E507G, S409M+L462M+E507L, S409M+L462S+E507A, S409M+L462S+E507M, S409M+L462S+E507S, S409M+L462S+E507G, S409M+L462S+E507L, S409M+L462G+E507A, S409M+L462G+E507M, S409M+L462G+E507S, S409M+L462G+E507G, S409M+L462G+E507L, S409G+L462A+E507A, S409G+L462A+E507M, S409G+L462A+E507S, S409G+L462A+E507G, S409G+L462A+E507L, S409G+L462M+E507A, S409G+L462M+E507M, S409G+L462M+E507S, S409G+L462M+E507G, S409G+L462M+E507L, S409G+L462S+E507A, S409G+L462S+E507M, S409G+L462S+E507S, S409G+L462S+E507G, S409G+L462S+E507L, S409G+L462G+E507A, S409G+L462G+E507M, S409G+L462G+E507S, S409G+L462G+E507G, S409G+L462G+E507L, S409L+L462A+E507A, S409L+L462A+E507M, S409L+L462A+E507S, S409L+L462A+E507G, S409L+L462A+E507L, S409L+L462M+E507A, S409L+L462M+E507M, S409L+L462M+E507S, S409L+L462M+E507G, S409L+L462M+E507L, S409L+L462S+E507A, S409L+L462S+E507M, S409L+L462S+E507S, S409L+L462S+E507G, S409L+L462S+E507L, S409L+L462G+E507A, S409L+L462G+E507M, S409L+L462G+E507S, S409L+L462G+E507G and S409L+L462G+E507L.

In another particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the combination of four substitutions at positions 409, 462, 507 and 629. In a particular embodiment, said human FVIII variant or biologically active derivative thereof comprises the combination of four substitutions selected from the group consisting of S409A+L462A+E507A+V629A, S409A+L462A+E507A+V629M,

S409A+L462A+E507A+V629S, S409A+L462A+E507A+V629G, S409A+L462A+E507A+V629L, S409A+L462A+E507M+V629A, S409A+L462A+E507M+V629M, S409A+L462A+E507M+V629S, S409A+L462A+E507M+V629G, S409A+L462A+E507M+V629L, S409A+L462A+E507S+V629A, S409A+L462A+E507S+V629M, S409A+L462A+E507S+V629S, S409A+L462A+E507S+V629G, S409A+L462A+E507S+V629L, S409A+L462A+E507G+V629A, S409A+L462A+E507G+V629M, S409A+L462A+E507G+V629S, S409A+L462A+E507G+V629G, S409A+L462A+E507G+V629L, S409A+L462A+E507L+V629A, S409A+L462A+E507L+V629M, S409A+L462A+E507L+V629S, S409A+L462A+E507L+V629G, S409A+L462A+E507L+V629L, S409A+L462M+E507A+V629A, S409A+L462M+E507A+V629M, S409A+L462M+E507A+V629S, S409A+L462M+E507A+V629G, S409A+L462M+E507A+V629L, S409A+L462M+E507M+V629A, S409A+L462M+E507M+V629M, S409A+L462M+E507M+V629S, S409A+L462M+E507M+V629G, S409A+L462M+E507M+V629L, S409A+L462M+E507S+V629A, S409A+L462M+E507S+V629M, S409A+L462M+E507S+V629S, S409A+L462M+E507S+V629G, S409A+L462M+E507S+V629L, S409A+L462M+E507G+V629A, S409A+L462M+E507G+V629M, S409A+L462M+E507G+V629S, S409A+L462M+E507G+V629G, S409A+L462M+E507G+V629L, S409A+L462M+E507L+V629A, S409A+L462M+E507L+V629M, S409A+L462M+E507L+V629S, S409A+L462M+E507L+V629G, S409A+L462M+E507L+V629L, S409A+L462S+E507A+V629A, S409A+L462S+E507A+V629M, S409A+L462S+E507A+V629S, S409A+L462S+E507A+V629G, S409A+L462S+E507A+V629L, S409A+L462S+E507M+V629A, S409A+L462S+E507M+V629M, S409A+L462S+E507M+V629S, S409A+L462S+E507M+V629G, S409A+L462S+E507M+V629L, S409A+L462S+E507S+V629A, S409A+L462S+E507S+V629M, S409A+L462S+E507S+V629S, S409A+L462S+E507S+V629G, S409A+L462S+E507S+V629L, S409A+L462S+E507G+V629A, S409A+L462S+E507G+V629M, S409A+L462S+E507G+V629S, S409A+L462S+E507G+V629G, S409A+L462S+E507G+V629L, S409A+L462S+E507L+V629A, S409A+L462S+E507L+V629M, S409A+L462S+E507L+V629S, S409A+L462S+E507L+V629G, S409A+L462S+E507L+V629L, S409A+L462G+E507A+V629A, S409A+L462G+E507A+V629M, S409A+L462G+E507A+V629S, S409A+L462G+E507A+V629G, S409A+L462G+E507A+V629L, S409A+L462G+E507M+V629A, S409A+L462G+E507M+V629M, S409A+L462G+E507M+V629S, S409A+L462G+E507M+V629G, S409A+L462G+E507M+V629L, S409A+L462G+E507S+V629A, S409A+L462G+E507S+V629M, S409A+L462G+E507S+V629S, S409A+L462G+E507S+V629G, S409A+L462G+E507S+V629L, S409A+L462G+E507G+V629A, S409A+L462G+E507G+V629M, S409A+L462G+E507G+V629S, S409A+L462G+E507G+V629G, S409A+L462G+E507G+V629L, S409A+L462G+E507L+V629A, S409A+L462G+E507L+V629M, S409A+L462G+E507L+V629S, S409A+L462G+E507L+V629G, S409A+L462G+E507L+V629L, S409M+L462A+E507A+V629A, S409M+L462A+E507A+V629M, S409M+L462A+E507A+V629S, S409M+L462A+E507A+V629G, S409M+L462A+E507A+V629L, S409M+L462A+E507M+V629A, S409M+L462A+E507M+V629M, S409M+L462A+E507M+V629S, S409M+L462A+E507M+V629G, S409M+L462A+E507M+V629L, S409M+L462A+E507S+V629A, S409M+L462A+E507S+V629M, S409M+L462A+E507S+V629S, S409M+L462A+E507S+V629G, S409M+L462A+E507S+V629L, S409M+L462A+E507G+V629A, S409M+L462A+E507G+V629M, S409M+L462A+E507G+V629S, S409M+L462A+E507G+V629G, S409M+L462A+E507G+V629L, S409M+L462A+E507L+V629A, S409M+L462A+E507L+V629M, S409M+L462A+E507L+V629S, S409M+L462A+E507L+V629G, S409M+L462A+E507L+V629L, S409M+L462M+E507A+V629A, S409M+L462M+E507A+V629M, S409M+L462M+E507A+V629S, S409M+L462M+E507A+V629G, S409M+L462M+E507A+V629L, S409M+L462M+E507M+V629A, S409M+L462M+E507M+V629M, S409M+L462M+E507M+V629S, S409M+L462M+E507M+V629G, S409M+L462M+E507M+V629L, S409M+L462M+E507S+V629A, S409M+L462M+E507S+V629M, S409M+L462M+E507S+V629S, S409M+L462M+E507S+V629G, S409M+L462M+E507S+V629L, S409M+L462M+E507G+V629A, S409M+L462M+E507G+V629M, S409M+L462M+E507G+V629S, S409M+L462M+E507G+V629G, S409M+L462M+E507G+V629L, S409M+L462M+E507L+V629A, S409M+L462M+E507L+V629M, S409M+L462M+E507L+V629S, S409M+L462M+E507L+V629G, S409M+L462M+E507L+V629L, S409M+L462S+E507A+V629A, S409 M+L462S+E507A+V629M, S409M+L462S+E507A+V629S, S409M+L462S+E507A+V629G, S409M+L462S+E507A+V629L, S409M+L462S+E507M+V629A, S409M+L462S+E507M+V629M, S409M+L462S+E507M+V629S, S409M+L462S+E507M+V629G, S409M+L462S+E507M+V629L, S409M+L462S+E507S+V629A, S409M+L462S+E507S+V629M, S409M+L462S+E507S+V629S, S409M+L462S+E507S+V629G, S409M+L462S+E507S+V629L, S409M+L462S+E507G+V629A, S409M+L462S+E507G+V629M, S409M+L462S+E507G+V629S, S409M+L462S+E507G+V629G, S409M+L462S+E507G+V629L, S409M+L462S+E507L+V629A, S409M+L462S+E507L+V629M, S409M+L462S+E507L+V629S, S409M+L462S+E507L+V629G, S409M+L462S+E507L+V629L, S409M+L462G+E507A+V629A, S409M+L462G+E507A+V629M, S409M+L462G+E507A+V629S, S409M+L462G+E507A+V629G, S409M+L462G+E507A+V629L, S409M+L462G+E507M+V629A, S409M+L462G+E507M+V629M, S409M+L462G+E507M+V629S, S409M+L462G+E507M+V629G, S409M+L462G+E507M+V629L, S409M+L462G+E507S+V629A, S409M+L462G+E507S+V629M, S409M+L462G+E507S+V629S, S409M+L462G+E507S+V629G, S409M+L462G+E507S+V629L, S409M+L462G+E507G+V629A, S409M+L462G+E507G+V629M, S409M+L462G+E507G+V629S, S409M+L462G+E507G+V629G, S409M+L462G+E507G+V629L, S409M+L462G+E507L+V629A, S409M+L462G+E507L+V629M, S409M+L462G+E507L+V629S, S409M+L462G+E507L+V629G, S409M+L462G+E507L+V629L, S409G+L462A+E507A+V629A, S409G+L462A+E507A+V629M, S409G+L462A+E507A+V629S, S409G+L462A+E507A+V629G, S409G+L462A+E507A+V629L, S409G+L462A+E507M+V629A, S409G+L462A+E507M+V629M, S409G+L462A+E507M+V629S, S409G+L462A+E507M+V629G, S409G+L462A+E507M+V629L, S409G+L462A+E507S+V629A, S409G+L462A+E507S+V629M, S409G+L462A+E507S+V629S, S409G+L462A+E507S+V629G, S409G+L462A+E507S+V629L, S409G+L462A+E507G+V629A, S409G+L462A+E507G+V629M, S409G+L462A+E507G+V629S, S409G+L462A+E507G+V629G, S409G+L462A+E507G+V629L, S409G+L462A+E507L+V629A, S409G+L462A+E507L+V629M, S409G+L462A+E507L+V629S, S409G+L462A+E507L+V629G, S409G+L462A+E507L+V629L, S409G+L462M+E507A+V629A, S409G+L462M+E507A+V629M, S409G+L462M+E507A+V629S, S409G+L462M+E507A+V629G, S409G+L462M+E507A+V629L, S409G+L462M+E507M+V629A, S409G+L462M+E507M+V629M, S409G+L462M+E507M+V629S, S409G+

L462M+E507M+V629G, S409G+L462M+E507M+V629L, S409G+L462M+E507S+V629A, S409G+L462M+E507S+V629M, S409G+L462M+E507S+V629S, S409G+L462M+E507S+V629G, S409G+L462M+E507S+V629L, S409G+L462M+E507G+V629A, S409G+L462M+E507G+V629M, S409G+L462M+E507G+V629S, S409G+L462M+E507G+V629G, S409G+L462M+E507G+V629L, S409G+L462M+E507L+V629A, S409G+L462M+E507L+V629M, S409G+L462M+E507L+V629S, S409G+L462M+E507L+V629G, S409G+L462M+E507L+V629L, S409G+L462S+E507A+V629A, S409G+L462S+E507A+V629M, S409G+L462S+E507A+V629S, S409G+L462S+E507A+V629G, S409G+L462S+E507A+V629L, S409G+L462S+E507M+V629A, S409G+L462S+E507M+V629M, S409G+L462S+E507M+V629S, S409G+L462S+E507M+V629G, S409G+L462S+E507M+V629L, S409G+L462S+E507S+V629A, S409G+L462S+E507S+V629M, S409G+L462S+E507S+V629S, S409G+L462S+E507S+V629G, S409G+L462S+E507S+V629L, S409G+L462S+E507G+V629A, S409G+L462S+E507G+V629M, S409G+L462S+E507G+V629S, S409G+L462S+E507G+V629G, S409G+L462S+E507G+V629L, S409G+L462S+E507L+V629A, S409G+L462S+E507L+V629M, S409G+L462S+E507L+V629S, S409G+L462S+E507L+V629G, S409G+L462S+E507L+V629L, S409G+L462G+E507A+V629A, S409G+L462G+E507A+V629M, S409G+L462G+E507A+V629S, S409G+L462G+E507A+V629G, S409G+L462G+E507A+V629L, S409G+L462G+E507M+V629A, S409G+L462G+E507M+V629M, S409G+L462G+E507M+V629S, S409G+L462G+E507M+V629G, S409G+L462G+E507M+V629L, S409G+L462G+E507S+V629A, S409G+L462G+E507S+V629M, S409G+L462G+E507S+V629S, S409G+L462G+E507S+V629G, S409G+L462G+E507S+V629L, S409G+L462G+E507G+V629A, S409G+L462G+E507G+V629M, S409G+L462G+E507G+V629S, S409G+L462G+E507G+V629G, S409G+L462G+E507G+V629L, S409G+L462G+E507L+V629A, S409G+L462G+E507L+V629M, S409G+L462G+E507L+V629S, S409G+L462G+E507L+V629G, S409G+L462G+E507L+V629L, S409L+L462A+E507A+V629A, S409L+L462A+E507A+V629M, S409L+L462A+E507A+V629S, S409L+L462A+E507A+V629G, S409L+L462A+E507A+V629L, S409L+L462A+E507M+V629A, S409L+L462A+E507M+V629M, S409L+L462A+E507M+V629S, S409L+L462A+E507M+V629G, S409L+L462A+E507M+V629L, S409L+L462A+E507S+V629A, S409L+L462A+E507S+V629M, S409L+L462A+E507S+V629S, S409L+L462A+E507S+V629G, S409L+L462A+E507S+V629L, S409L+L462A+E507G+V629A, S409L+L462A+E507G+V629M, S409L+L462A+E507G+V629S, S409L+L462A+E507G+V629G, S409L+L462A+E507G+V629L, S409L+L462A+E507L+V629A, S409L+L462A+E507L+V629M, S409L+L462A+E507L+V629S, S409L+L462A+E507L+V629G, S409L+L462A+E507L+V629L, S409L+L462M+E507A+V629A, S409L+L462M+E507A+V629M, S409L+L462M+E507A+V629S, S409L+L462M+E507A+V629G, S409L+L462M+E507A+V629L, S409L+L462M+E507M+V629A, S409L+L462M+E507M+V629M, S409L+L462M+E507M+V629S, S409L+L462M+E507M+V629G, S409L+L462M+E507M+V629L, S409L+L462M+E507S+V629A, S409L+L462M+E507S+V629M, S409L+L462M+E507S+V629S, S409L+L462M+E507S+V629G, S409L+L462M+E507S+V629L, S409L+L462M+E507G+V629A, S409L+L462M+E507G+V629M, S409L+L462M+E507G+V629S, S409L+L462M+E507G+V629G, S409L+L462M+E507G+V629L, S409L+L462M+E507L+V629A, S409L+L462M+E507L+V629M, S409L+L462M+E507L+V629S, S409L+L462M+E507L+V629G, S409L+L462M+E507L+V629L, S409L+L462S+E507A+V629A, S409L+L462S+E507A+V629M, S409L+L462S+E507A+V629S, S409L+L462S+E507A+V629G, S409L+L462S+E507A+V629L, S409L+L462S+E507M+V629A, S409L+L462S+E507M+V629M, S409L+L462S+E507M+V629S, S409L+L462S+E507M+V629G, S409L+L462S+E507M+V629L, S409L+L462S+E507S+V629A, S409L+L462S+E507S+V629M, S409L+L462S+E507S+V629S, S409L+L462S+E507S+V629G, S409L+L462S+E507S+V629L, S409L+L462S+E507G+V629A, S409L+L462S+E507G+V629M, S409L+L462S+E507G+V629S, S409L+L462S+E507G+V629G, S409L+L462S+E507G+V629L, S409L+L462S+E507L+V629A, S409L+L462S+E507L+V629M, S409L+L462S+E507L+V629S, S409L+L462S+E507L+V629G, S409L+L462S+E507L+V629L, S409L+L462G+E507A+V629A, S409L+L462G+E507A+V629M, S409L+L462G+E507A+V629S, S409L+L462G+E507A+V629G, S409L+L462G+E507A+V629L, S409L+L462G+E507M+V629A, S409L+L462G+E507M+V629M, S409L+L462G+E507M+V629S, S409L+L462G+E507M+V629G, S409L+L462G+E507M+V629L, S409L+L462G+E507S+V629A, S409L+L462G+E507S+V629M, S409L+L462G+E507S+V629S, S409L+L462G+E507S+V629G, S409L+L462G+E507S+V629L, S409L+L462G+E507G+V629A, S409L+L462G+E507G+V629M, S409L+L462G+E507G+V629S, S409L+L462G+E507G+V629G, S409L+L462G+E507G+V629L, S409L+L462G+E507L+V629A, S409L+L462G+E507L+V629M, S409L+L462G+E507L+V629S, S409L+L462G+E507L+V629G, S409L+L462G+E507L+V629L.

In a further preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having an improved specific activity and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain. Said variant can further comprise the substitution of the amino acid at position 2196 of the C2 domain. Moreover, said mutations which confer a higher specific activity may prove to be of great interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of activity of said less antigenic mutants. Thus, in a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain, and further comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain. Preferably, said variant comprises the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312 and 2316 of the C2 domain, and further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of C2 the domain.

In an additional preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having an improved capacity to be secreted and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2199, 2200, 2215, 2251, 2252, 2278, and 2316 of the C2 domain. Said variant can further comprise the substitution of the amino acid at position 2175 of the C2 domain. Furthermore, said mutations which confer higher capacity to be secreted may prove to be of great interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants. Thus, in a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain, and further comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain. In a preferred manner, said variant comprises the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312 and 2316 of the C2 domain, and further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain.

The broad production of mutants having retained at least 50% of FVIII activity also makes it possible to encompass their use in the context of analyzing additional functions of the protein. In addition to a modulation of its immunogenicity, secretion and specific activity, the following FVIII properties might be improved by using the mutants molecules described: —binding to von Willebrand factor and therefore improved half-life of FVIII or circulating FVIIIa; —improved intrinsic stability of the molecule by stabilization of the A2 domain and therefore increase of its efficiency period; —binding to phospholipids derived from blood platelets, cell surfaces or circulating microparticles and therefore improved generation of FXa; —binding to FIXa and FX and therefore improved formation of FXa; —decreased binding of FVIII or FVIIIa to the molecules responsible of its catabolism such as for example low density Lipoprotein Receptor-related Protein (LRP), Low Density Lipoprotein Receptor (LDLR), Very Low Density Lipoprotein Receptor (VLDLR), megaline or any other receptor which might be identified and therefore improvement of the circulating FVIII half-life of; —decreased proteolysis of FVIII or FVIIIa by vascular proteases such as for example activated protein C, FXa, FIXa, and therefore increased of its efficiency period.

Preferably, the biologically active FVIII derivative is a FVIII consisting in a whole or partial deletion of the B domain. The human FVIII variant of the present invention is not a hybrid FVIII. It does not contain a substitution of the A2 or C2 domain or of a segment of at least 15 consecutive amino acids thereof by a FVIII domain of another species. In particular, segments of the A2 domain 373-540, 373-508, 445-508, 484-508, 404-508, 489-508 and/or 484-489 are not substituted by those of another species. In a particular embodiment, the polypeptide sequence of the variant differs from that of human FVIII such as described in SEQ ID No. 3 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 substitutions, preferably by 1, 2, 3, 4, 5, 6, 7 or 8 substitutions, without including an optional deletion or truncation. In a particular embodiment, the variant comprises a single substitution. In another particular embodiment, the variant comprises a combination of 1 to 8 substitutions selected from a group according to the present invention.

"Inhibitory antibodies" or "inhibitors" refers to any antibody which recognizes or binds to FVIII and inhibits the biological activity thereof, in particular the procoagulant activity thereof. In particular, said antibodies can preferably recognize i) the C2 domain of the light chain (2181-2321); ii) the A2 domain of the heavy chain (484-509); or iii) the A3 domain (1694-2019). Examples of commercially available inhibitory antibodies comprise ESH-8 (strong inhibitor; recognized the region 2248/2285; 6300 BU/mg; anti-C2; America Diagnostica), GMA-8015 (anti-A2; Green Mountain), anti-C2 ESH-4 antibody (strong inhibitor; region 2303/2332; America Diagnostica), anti-C2 Bo2C11 antibody (Jacquemin et al., 1998, Blood, 92(2):496-506).

"Patients with inhibitors" are patients who have FVIII inhibitory antibodies in their serum. The recognition profile of said antibodies differs from a patient to another. An improved FVIII according to the present invention is a FVIII which at least partially avoids one or more types of inhibitory antibodies.

"Biologically active derivative of FVIII" refers to any protein or peptide derived from human FVIII which retains a procoagulant activity of FVIII. For example, such biologically active FVIII derivative may be a FVIII whose B domain (741-1648) has been partially or totally deleted (Toole et al., 1986, Proc. Natl. Acad. Sci. USA, 83 (16):5939-5942; Pittman, 1993, Blood, 81:2925-2935; Eaton et al., 1986, Biochemistry, 25 (26):8343-8347; Langer et al., 1988, Behring Inst. Mitt, 82:16-25; Meulien et al., 1988, Protein Eng, 2(4): 301-6; and U.S. Pat. No. 4,868,112). Moreover, this term also refers to FVIII mutants with a stabilized A2 domain (WO 97/40145), FVIII mutants allowing a higher expression (Swaroop et al., 1997, JBC, 272:24121-24124), FVIII mutants having decreased antigenicity (Lollar, 1999, Thromb. Haemost. 82:505-508), a FVIII reconstituted from separately expressed light and heavy chains (Oh et al., 1999, Exp. Mol. Med. 31:95-100), FVIII mutants displaying decreased binding to FVIII catabolic associated receptors such as HSPG (heparan sulfate proteoglycans) and LRP (low density lipoprotein receptor related protein) (Ananyeva et al., 2001, TCM, 11:251-257), FVIII mutants displaying an improved specific activity (US2004/0249134). Also considered are FVIII variants in which FVIII segments are replaced by the corresponding segments of factor V (Marquette et al., 1995, JBC, 270:10297-10303, Oertel et al., 1996, Thromb. Haemost., 75:36-44). Moreover, said term refers to any FVIII comprising one or more substitutions, deletions or insertions. For example, it comprises the variants described in the introduction of the present application, in particular those comprising point mutations. In particular, it comprises a FVIII less susceptible to cleavage by APC (activated protein C) comprising mutations of Arginines 336 and 562 and in the region comprised between the positions 2001-2020, as described in application WO 2006/027111. It further comprises a stabilized FVIII mutant in which one or more Cysteines have been introduced so as to create one or more disulfide bonds, for example between the A2 and A3 domains (WO02103024; Gale et Pellequer, 2003, J Thromb Haemost, 1(9):1966-71). Patents JP2005112855 and RU2244556/RU2253475 respectively provide biologically stable and albumin-free compositions, allowing the stabilization of FVIII alone or in association with vWF. This term also refers to any FVIII having been modified by conjugation of a functional group, for example PEGylation, glycosylation (for example US2005009148, US2003077752, etc.). Furthermore, the variant can comprise peptide bonds modified in order to resist to hydrolysis.

In particular, the variant has a decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII and retains a procoagulant activity at least equal to 50% that of natural human FVIII. For example, one suitable assay is the one or two-stage clotting assay described in Rizza et al. (Rizza et al., 1982, Coagulation assay of Factor VIIIa and FIXa in Bloom ed. The Hemophilias. NY Churchchill Livingston 1992). In a preferred embodiment, the variant retains a procoagulant activity equal to that of natural human FVIII. In a more preferred embodiment, the variant has a procoagulant activity higher than that of natural human FVIII.

The procoagulant activity of FVIII is determined by any method known to one skilled in the art. Preferably, said procoagulant activity is determined by chronometric assay or by chromogenic assay. Even more preferably, FVIII activity is determined by chronometric assay, for example as described by Von Clauss (A. Acta Haematologica, 1957, 17:237) or by chronometric assay such as described by Rosen (Scand. J. Haematol. 1984, 33 (Suppl 40):139-145).

The present invention relates to a nucleic acid coding for a human FVIII variant according to the invention. The present invention also relates to an expression cassette of a nucleic acid according to the invention. It further relates to a vector comprising a nucleic acid or an expression cassette according to the invention. The vector can be selected from a plasmid and a viral vector.

The nucleic acid can be DNA (cDNA or gDNA), RNA, or a mixture of the two. It can be in single stranded form or in duplex form or a mixture of the two. It can comprise modified nucleotides, comprising for example a modified bond, a modified purine or pyrimidine base, or a modified sugar. It can be prepared by any method known to one skilled in the art, including chemical synthesis, recombination, mutagenesis etc.

The expression cassette comprises all elements required for expression of the human FVIII variant according to the invention, in particular the elements required for transcription and translation in the host cell. The host cell can be prokaryotic or eukaryotic. In particular, the expression cassette comprises a promoter and a terminator, optionally an enhancer. The promoter can be prokaryotic or eukaryotic. Examples of preferred prokaryotic promoters include: LacI, LacZ, pLacT, ptac, pARA, pBAD, the RNA polymerase promoters of bacteriophage T3 or T7, the polyhedrin promoter, the PR or PL promoter of lambda phage. Examples of preferred eukaryotic promoters include: CMV early promoter, HSV thymidine kinase promoter, SV40 early or late promoter, mouse metallothionein-L promoter, and the LTR regions of some retroviruses. In general, to select a suitable promoter, one skilled in the art may advantageously consult Sambrook et al. work (1989) or techniques described by Fuller et al. (1996; Immunology in Current Protocols in Molecular Biology).

The present invention relates to a vector containing a nucleic acid or an expression cassette coding for a human FVIII variant according to the invention. The vector is preferably an expression vector, that is to say, it comprises the elements required for the expression of the variant in the host cell. The host cell can be a prokaryote, for example *E. coli*, or a eukaryote. The eukaryote can be a lower eukaryote such as a yeast (for example, *S. cerevisiae*) or fungus (for example from the genus *Aspergillus*) or a higher eukaryote such as an insect, mammalian or plant cell. The cell can be a mammalian cell, for example COS, CHO (U.S. Pat. Nos. 4,889,803 ; 5,047,335). In a particular embodiment, the cell is non-human and non-embryonic. The vector can be a plasmid, phage, phagemid, cosmid, virus, YAC, BAC, pTi plasmid from *Agrobacterium*, etc. The vector can preferably comprise one or more elements selected from the group consisting of a replication origin, a multiple cloning site and a selection gene. In a preferred embodiment, the vector is a plasmid. Examples of prokaryotic vectors include, but are not limited to, the following: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pBR322, and pRIT5 (Pharmacia), pET (Novagen). Examples of eukaryotic vectors include, but are not limited to, the following: pWLNEO, pSV2CAT, pPICZ, pcDNA3.1 (+) Hyg (Invitrogen), pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pCI-neo (Stratagene), pMSG, pSVL (Pharmacia); and pQE-30 (QLAexpress). Examples of viral vectors include, but are not limited to, adenoviruses, AAV, HSV, lentiviruses, etc. Preferably the expression vector is a plasmid or a viral vector.

The coding sequence for FVIII according to the present invention can comprise or not comprise the signal peptide. In the case where coding sequence does not comprise signal peptide, a methionine can optionally be added at the N-terminal end. Alternatively, a heterologous signal peptide can be introduced. Said heterologous signal peptide can be derived from a prokaryote such as *E. coli* or from a eukaryote, in particular from a mammalian, insect or yeast cell. Moreover, the nucleotide sequence can also comprise intron segments, particularly heterologous introns. Said intron segments can enable improved expression of the FVIII variant. Such constructs are described in application WO 2005/040213. For example, the nucleotide sequence can comprise modified sequence SEQ ID No. 5 so as to code for the FVIII variant comprising the substitution or substitutions according to the present invention.

The present invention relates to the use of a nucleic acid, an expression cassette or a vector according to the invention in order to transform or transfect a cell. The invention relates to a host cell comprising a nucleic acid, an expression cassette or a vector coding for a human FVIII variant and the use thereof to produce a recombinant human FVIII variant according to the invention. In a particular embodiment, the cell is non-human and non-embryonic. The invention also relates to a method for producing a recombinant human FVIII variant according to the invention comprising transforming or transfecting a cell by a nucleic acid, an expression cassette or a vector according to the invention; culturing the transformed/transfected cell; and collecting the human FVIII variant produced by the cell. In an alternative embodiment, the method for producing a recombinant human FVIII variant according to the invention comprises providing a cell comprising a nucleic acid, an expression cassette or a vector according to the invention; culturing the transfected/transformed cell; and collecting the human FVIII variant produced by the cell. In particular, the cell can be transformed/transfected in a transient or stable manner by the nucleic acid coding for the variant. Said nucleic acid can be contained in the cell in an episome form of or in chromosomal form. Method for producing recombinant proteins are well known to one skilled in the art. For example, one can mention the specific method described in WO0170968 for a production in an immortalized human cell line, WO2005/123928 for production in a plant, US2005/229261 for production in the milk of a transgenic animal, etc.

The present invention relates to pharmaceutical compositions comprising human FVIII variants according to the invention, and to the use of said FVIII variants for preparing a medicament for the treatment of hemophilia A. Preferably, the hemophilia A is severe and moderate. Said treatment can be curative or preventive. In a particular embodiment, the treated patients are patients with inhibitors.

Thus, the FVIII variants according to the invention can be used in two major categories of hemophiliac patients: those who have developed FVIII inhibitory antibodies, thanks to their capacity to avoid said inhibitory antibodies, and those who have not yet developed such inhibitors, thanks to their lower risk of inducing the development of inhibitory antibodies as compared to the molecules currently used. Said FVIII variants will be usable by all patients with hemophilia A.

The present invention therefore relates to a pharmaceutical composition comprising a FVIII variant according to the invention. The pharmaceutical composition can further comprise compounds for stabilizing the mutant FVIII, for example serum albumin, vWF (von Willebrand factor) or a fragment thereof comprising the FVIII binding site, vitamin K-dependent coagulation factors, and polysaccharides such as sucrose. The present invention can also relate to a pharmaceutical composition comprising a nucleic acid coding for a FVIII mutant according to the invention, a vector or a host cell according to the invention. Such composition might be useful in the context of a gene therapy. The pharmaceutical composition can further comprise a pharmaceutically acceptable excipient or carrier. Such excipients and carriers are well known to one skilled in the art [Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company (1990); Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis (2000); and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000)] and comprise physiological saline solutions and phosphate buffers. The FVIII variant according to the invention can also be formulated in a pharmaceutical composition with phospholipids or equivalents, for example in the form of liposomes, nanoparticles, etc. (WO2004/071420; WO2004/091723). The pharmaceutical composition can further comprise one or more other active ingredients.

The present invention also relates to a FVIII variant according to the invention as medicament. It further relates to a nucleic acid coding for a FVIII mutant, an expression cassette, a vector or a host cell according to the invention, as medicament.

The human FVIII variants of the invention can be used as replacement therapy in case of severe and moderate hemophilia A. The possibility of a continuously use with a lower risk of developing inhibitory antibodies is a major advantage over the different existing recombinant human or hybrid FVIIIs.

Said improved human FVIII variants are preferably intended for treating patients who have already developed inhibitors, but also for preventive treatment.

In addition, systematic administration of said FVIII might be encompass for a prophylactic treatment in any patient with hemophilia A. One might therefore imagine decreasing the risks of bleeding, for example during surgical procedures, or else preventing the development of inhibitors. The administration of said FVIII might also be considered in the case of an emergency treatment, for example during an accidental, pathological hemorrhage or caused by a surgical procedure.

The pharmaceutical compositions of the invention are suitable for oral, sublingual, subcutaneous, intramuscular, intravenous, topical, local, intratracheal, intranasal, transdermal, rectal, intraocular, intra-auricular administration, said active ingredient being able to be administered as a unit dose. Preferably, the pharmaceutical compositions are suitable for intravenous, subcutaneous or intramuscular administration.

The dosages of the treatment can differ according to the severity of FVIII deficiency. Usually, the dosage is adjusted for frequency, period and units related to the severity and length of the bleeding episodes of the considered patient. FVIII is dosed so as to arrest bleeding, for example with standard clotting assays. An efficient dose of FVIII variant according to the invention can comprise, but is not limited to, between about 5 to 50 units per kg of body weight, preferably between 10 to 50, even more preferably between 20 to 40. The dosing frequency can be for example every 8 to 24 hours. The treatment duration can be for example from 1 to 10 days, or until bleeding stops. [See for example: Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions—Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII), "Ch. 153, 1453-1474,1460, in Hematology, Williams, W. J. et al., ed. (1990)].

The treatment can be in the form of a single intravenous injection or periodic or continuous administration over an extended period of time, as necessary. The treatment can also be administered by the subcutaneous or oral route with liposomes in one or more doses at different time intervals.

The present invention relates to the use of a human FVIII variant or a biologically active derivative thereof according to the invention for preparing a medicament for the treatment of coagulation disorders, in particular hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. The present invention also relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the invention.

The present invention further relates to the use of a nucleic acid coding for a FVIII variant according to the invention for preparing a medicament for the treatment of coagulation disorders, in particular hemophilia A.

The FVIII variant of the invention can also be combined with another active compound. For example, the present invention also relates to the use of a FVIII variant according to the invention in combination with factor IXa for treating coagulation disorders, and in particular hemophilia A or B. Said combination is described in WO2004/103397.

The present invention further relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the invention for the diagnosis of inhibitor type in a patient with hemophilia A. In particular, the presence of inhibitory antibodies is assayed in serum samples or biological fluids (lymph, urine, etc.). Detection of inhibitory antibodies can be carried out by ELISA, immunodetection by electrophoretic blotting, radioimmunoassay, and FVIII activity assays (for example, clotting assay).

In fact, inventors have identified in wild-type human FVIII the positions specifically recognized by the inhibitors. Said positions can be used individually, combined within a same domain, or combined between the A2 and C2 domains, so as to reveal the type(s) of inhibitory antibodies present in a hemophiliac. In fact, the need to diagnose inhibitory antibodies is crucial. The titration of said inhibitors is a prerequisite prior to any replacement therapy. The inventors therefore propose to use of the present findings to diagnose inhibitory antibodies. A Bethesda assay (assay of inhibitor titer) in a hemophiliac patient can be carried out before and after passage on ELISA where the capture antigen corresponds to the FVIII variants of the present invention taken separately or combined. The inhibitor titer will significantly decrease for the control carried out with wild-type FVIII. The variant or variants combination for which the inhibitor titer remains unchanged is used as treatment for the hemophiliac patient with inhibitors. This diagnosis therefore renders possible to control and target the delivery of the human FVIII variant according to the invention.

Thus, the present invention relates to a method for treatment comprising:

a recognition test of inhibitory antibodies contained in a serum sample of patient on one or more FVIII variants according to the invention;

selection of the FVIII mutant or mutants which are not recognized by said inhibitory antibodies; and

administration of one or more FVIII mutants selected from b).

In a preferred manner, the recognition test between the patient's sample and the FVIII variant(s) according to the invention is carried out by a Bethesda assay. As a control, a recognition test is preferably carried out on wild-type FVIII.

The present invention relates to a diagnostic kit comprising one or more FVIII variants according to the invention.

The present invention also relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the invention for preparing a medicament for the treatment of hemophilia A in patients with inhibitors whose serum does not contain antibodies recognizing said human FVIII variant(s) or a biologically active derivative thereof.

All references cited herein are included by reference in the present application. Other features and advantages of the invention will become apparent in the following examples which are provided for purposes of illustration and not by way of limitation.

EXAMPLES

Example 1

Molecular Biology

FVIII complementary DNA containing two truncated introns of factor IX at position 1 and 13 (5012 bp) (SEQ ID No. 4) was cloned between the NotI and Xho1 restriction sites in a vector (pcDNA3.1 GS, Invitrogen) allowing expression of the protein in mammalian cells. The pcDNA/FVIII construct corresponded to a 10,439 bp plasmid. This gene comprises the five functional domains A1, A2, A3, C1 and C2 essential for FVIII activity. As it has previously been shown that the B domain does not play any predominant role in the procoagulant function of FVIII, the inventors chose to produce FVIII with a deletion of this domain. The regions coding for the A1 and A2 domains each contain an intron. Insertion of these two intron regions among the coding exons significantly improves the expression of human FVIII. The protein sequence encoded by this gene is given in SEQ ID No. 5.

The mutagenesis strategy consisted in systematically generating all the single Alanine mutants in the targeted domains of FVIII, i.e., A2, A3 and C2. Said mutants were generated by the Massive Mutagenesis® method described in US2004/0048268.

As mentioned earlier, it has been shown that domains A2, C2 and A3 are the preferential targets of FVIII recognition by inhibitory antibodies. Each amino acid in these functional domains was substituted by an Alanine, apart from the intron segment of the A2 domain. A series of 795 oligonucleotides (32-mers) was designed and produced so as to introduce an Alanine mutation at positions i) 376 to 719 [A2]; ii) 2173 to 2325 [C2]; iii) 1691 to 2025 [A3]. The numbering system for the mutations of human FVIII used in the invention is that defined by Wood et al. (Nature, 1984, 312:330-337). After site-directed mutagenesis, the inventors performed two successive sequencings to check that each mutant of the library contained the Alanine mutation at the considered position. This collection of Alanine mutants in the C2, A2 and A3 domains of FVIII is the first comprehensive site-directed mutants library ever carried out for this molecule.

Example 2

Expression of Human FVIII Alanine Mutants in COS-7 Mammalian Cells

FVIII is usually expressed in mammalian cells (Toole et al., 1984, Nature, 312:342-347; Gitschier et al., 1984, Nature, 312:326-330; Wood et al., 1984, Nature, 312:330-337; Vehar et al., 1984, Nature, 312:337-342; WO8704187; WO 8808035; WO8803558; U.S. Pat. No. 4,757,006).

In order to transfect COS-7 cells with the native or mutated pcDNA/FVIII constructs, said cells were trypsinized when they reached 90% confluence. The COS-7 cells were reseeded at a 1/4 ratio (that is, in order to obtain approximately 25% confluence once they adhered to the surface). Transient transfection of COS-7 cells was carried out in 90 mm culture plates (6 ml per well) when cells reached 70-80% confluence. Transfection was carried out with approximately 6 μg DNA for a volume of 18 μl FuGENE-6 (Roche, Meylan, France).

Prior to transfection, FuGENE-6 was diluted in serum-free IMDM medium and incubated at room temperature for 5 min. The FuGENE-6/DNA mixture was left at room temperature for 15 min then deposited dropwise on the cells in complete medium. A first supernatant containing FVIII was collected 24 h after transfection; 6 ml of fresh medium were then placed on the cells. The culture supernatant was collected 48 h later (6 ml), aliquoted and stored at −20° C. pending the clotting assay (chromogenic). The mean level of expression of wild-type FVIII was estimated by ELISA (Stago commercial ELISA kit) and was comprised between 20 and 60 ng/ml.

All cell culture reagents were from Invitrogen. COS-7 cells (African green monkey SV40 transformed kidney cells) were grown in standard culture conditions (37° C. in a humid 5% CO_2 atmosphere) using Iscove's Modified Dulbecco's Medium (IMDM). IMDM was supplemented with an L-glutamine analog (glutamax), decomplemented fetal calf serum (10% final concentration) and antibiotics (penicillin 40 U/ml and streptomycin 0.1 mg/ml).

Example 3

Primary Screen: Functional Analysis of Human FVIII Alanine Mutants

The primary screen correlates to raw coagulant activity determination (FIG. **1**) obtained in a same volume of COS-7 cell culture supernatant. Two different assays of clotting activity determination were used in the primary screen, the chronometric assay and the chromogenic assay.

Chronometric activity was measured following incubation of a dilution of the FVIII molecules to be tested in imidazole buffer in the presence of FVIII-deficient plasma (Stago). Clotting was initiated by addition of calcium and the time to clot formation was determined on a MDA-II apparatus (BioMérieux, Marcy-l'Etoile). The coagulant activity of the 795 Alanine mutants was measured by chronometric assay on a robotic platform of the National Hemophilia Treatment

Center (Hospices Civils de Lyon). The chronometric activity of all the Alanine mutants was compared to the activity of a wild-type FVIII used as internal standard for each transfection. Results of these determinations of raw activity relative to that of non-mutated FVIII distinguished two categories of mutants: I) mutants having retained at least 50% of wild-type FVIII activity; ii) mutants having less than 50% of wild-type FVIII activity. FIG. **2** shows the coagulant activity of 359 over 795 Alanine mutants analyzed. These data represent a functional mapping of each of these FVIII residues for coagulant activity; a coagulant activity suppressed by an Alanine mutation indicates that the considered residue is essential for FVIII coagulant activity.

158 mutants having retained more than 50% of raw non-mutated FVIII activity were selected by this chronometric assay for secondary screen. Their activities were first confirmed by the second clotting assay, the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the 158 selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier's instructions. Briefly, culture supernatants (50 μl) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50 μl), preheated at 37° C., was then added for 4 min, after which 50 μl of development medium at 37° C. were added. The formation of product over time was measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

Table 1 shows the activities of the 158 mutants which retained more than 50% of non-mutated FVIII activity. Said 158 mutants were selected for the secondary screening.

Example 4

Secondary Screen: Evaluation of Loss of Antigenicity Towards Human FVIII Inhibitory Antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the 158 mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chronometric assay.

Culture supernatants obtained after 48 h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chronometric activity comprised in the range of about 10-20% (1 FVIII unit=100% activity=200 ng/ml). The culture supernatant diluted or not (140 μl) was added to 150 μl of FVIII-depleted human plasma (Stago, Asnières, France). An antibody dilution (10 μl) was then added to the mix. These antibodies are IgG fractions purified on protein A—from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained. Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1 h 30. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-I'Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient's serum. Said percentage was calculated as shown in FIG. **5** for the FVIII mutant E518A. Abolition to inhibition expressed is a percentage=−[(b−a)/a]×100; where "a" is the percentage residual activity of the WT (serum+IgG/serum−IgG) and "b" is the percentage residual activity of the mutant (serum+IgG/serum−IgG).

Table 2 shows for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant 2316 for sera TD and SL, mutant 2294 for serum GC, mutant 403 for serum FS and mutant 2275 for serum PR.

Patients' sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIIIs produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least 50% of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). Table 3 shows comparative data of raw and specific activities of 30 mutants selected in the secondary screen.

The eight FVIII Alanine mutants 2175, 2199, 2200, 2215, 2251, 2252, 2278 and 2316 displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. **3** depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15 mutants are given in FIG. **4**. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

Example 5

Selection and Combination of the Best Single Mutants Selected in the Secondary Screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophiliac patients with inhibitors;
- raw coagulant activity at least 100% relative to non-mutated FVIII; and
- reproducibly good level of expression.

The eight selected mutants were mutants 409, 462, 507 and 629 in the A2 domain and mutants 2289, 2294, 2312 and 2316 in the C2 domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in Table 3. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations 409, 462, 507, 629, 2289, 2294, 2312 and 2316 (six A2 double mutants+six C2 double mutants+sixteen A2-C2 double mutants presented in Table 4) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-7 mammalian cells as described in Example 2. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophiliac patients. The A2 double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C2 domain mutations (six C2 double mutants+sixteen A2-C2 double mutants) displayed an insignificant or null abolition to inhibition.

Table 5 shows the specific activities of the six A2 double mutants and their percentage of abolition to inhibition by sera from four hemophiliac patients TD, GC, SL and PR calculated as in Example 4. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A2 domain. The choice was therefore based on the combination of the four mutations 409, 507, 462 and 629. Triple mutants and the quadruple mutant comprising these four mutations 409, 507, 462 and 629 were also constructed.

Example 6

Construction and Characterization of a Quadruple Mutant (FVIII-4A2)

The quadruple mutant derived from the combination of the four selected A2 mutations 409, 462, 507, 629 was constructed by a classical mutagenesis method known to one skilled in the art. The quadruple mutant was produced in a CHO cell line obtained as described in Example 9. This mutant was also characterized for its abolition to inhibition by antibodies from five hemophiliac patients FS, TD, GC, PR and SL. Residual activity determined after incubation with an inhibitory antibody is divided by residual activity remaining after incubation with a non-immune antibody. The percentage of residual activity was thus determined and is presented in the graphs of FIG. **6**. These graphs illustrate the residual activity of FVIII-4A2 after contact with different dilutions of antibodies from the different patients with inhibitors. It clearly appears that the FVIII-4A2 mutant retained a much higher chronometric activity after incubation with the inhibitory antibodies. Accordingly, the increases in residual activity for the highest inhibitory antibodies concentrations ranged from 230 to 450%, said percentage of residual activity depending on both the source of the inhibitory antibody and the concentration used.

To determine whether direct binding of the antibodies to FVIII-4A2 was modified, three additional antibodies were used instead of the patients' sera according to the same protocol as above: an anti-A2 domain antibody (GMA012, Green Mountain Antibodies), an anti-C2 domain antibody (ESH4, American Diagnostica) and a rabbit polyclonal antibody, purified from the same protocol used for the patients' antibodies. The results of these controls are shown in FIG. **7** for the two anti-A2 domain antibodies, the rabbit polyclonal antibody and GMA012. Clearly, the mutations in the A2 domain of FVIII-4A2 allowed FVIII-4A2 to avoid the anti-A2 domain antibody, GMA012 and the rabbit polyclonal antibody (shown). On the other hand, no significant differences in inhibition of FVIII-4A2 versus wild-type FVIII were seen for ESH4 (data not shown). These findings correlate the abolition to inhibition data, showing on one hand that introduction of mutations in the A2 domain allow to avoid patients' antibodies and on the other hand that the C2 domain of FVIII-4A2 is undamaged since recognition is similar to that of wild-type FVIII. This latter point is important for FVIII-4A2 activity because it is the C2 domain which is responsible for interactions with von Willebrand factor and with the cofactors required for full FVIII activity (calcium and phospholipid binding).

Example 7

Characterization of the FVIII 4A2 Mutant

a) ELISA

FVIII-4A2 was produced in the same CHO cell line as wild-type FVIII according to the protocol described in Example 9. It was purified by the same protocol (also described in Example 9) and was therefore compared to FVIII in functional analyses. FVIII-4A2 concentrations were determined with an ELISA kit (see protocol below). Additional controls were performed using a panel of monoclonal antibodies to check that the introduced mutations did not alter the quantification of mutant FVIII with this kit. Thereby, it was shown that similar concentrations of wild-type FVIII and FVIII-4A2 were identically recognized by antibody ESH-4 directed against the light chain C2 domain. In agreement with the abolition to inhibition data, there was a large decrease in recognition of FVIII-4A2 by the GMA012 antibody in comparison with wild-type FVIII. These data are presented in FIG. **8**.

The protocol of the ELISA assays for these experiments is described below:

Reagent was diluted at least five-fold in 50 mM CAPS pH 9.0 and incubated overnight at 4° C. to coat the interest product on the support of the ELISA plate (Nunc Maxisorb). Wells were then washed twice with TBS-T buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween 20, 0.05% BSA), then blocked for 1 h with TBS-3% BSA (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween-20, 3% BSA). Reagent binding with the one coated on the plate was then diluted in TBS-3% BSA, incubated at room temperature for 1 h 30, then washed three times in TBS-T. Primary and secondary antibodies conjugated to horse radish peroxidase (HRP) were diluted in TBS-3% and respectively added for 1 h 30 at room temperature. Secondary antibodies were diluted 2000-fold. Between two antibody incubations, plates were washed three times with TBS-T, then washed again before addition of the substrate, a mixture of OPD/urea (Sigma). The enzymatic reaction was stopped by adding 2.5M H_2SO_4. Optical density was read at 490 nm.

b) Measurement of Specific Activity

Specific activity of the FVIII-4A2 mutant was determined by dividing chromogenic activity by concentration. These specific activities were compared with those of the wild-type. The chromogenic activity of wild-type FVIII was about 15±1 ODU/min·μg and that of FVIII-4A2 was about 27±1 ODU/min·μg, that is, a higher activity.

c) Activation by Thrombin

Wild-type FVIII and FVIII-4A2 (0.125 U or 25 ng) were diluted in 40 mM HEPES buffer, 100 mM NaCl, 5 mM $CaCl_2$ containing 10 μM of an 80:20 mixture of Phosphatidylcholine:Phosphatidylserine and 0.1 mg/ml BSA, then incubated at 37° C. for 5 min. Thrombin (0.05 U) was added and its action determined at different time. At each time, an aliquot was removed and incubated with a mixture of hirudin (0.5 U), factor IXa (50 nM) and factor X (200 nM) diluted in the same buffer, in order to generate FXa. The FXa substrate pNAPEP-25 was immediately added and formation of the chromogenic product was measured at 405 nm. The initial rate was determined and the amount of FXa formed per minute was calculated.

Wild-type FVIII and FVIII-4A2 displayed an identical thrombin response profile, with a rapid increase in FVIII activity, reaching the peak at 1-2 min after addition of thrombin, followed by a rapid decrease of said activity with a half-life of approximately 2-3 min. The results shown in FIG. **9** indicate that FVIII-4A2 is identically recognized by thrombin as wild-type FVIII with a relative decrease of activity which might be caused by one of the four mutations.

d) Dissociation of the A2 Domain

Wild-type FVIII and FVIII-4A2 were activated as described above for 1 min. Hirudin was then added and FVIIIa was left at 37° C. for different time periods. Aliquots were removed at said time and incubated with a mixture of phospholipids, FIXa and FX. FXa was allowed to form for 5 min, then Stop buffer was added (Iris 50 mM pH 8.8, 475 mM NaCl, 9 mM EDTA). The amount of FXa formed was determined as above.

FVIIIa was incubated for different times before determining its residual activity. The loss of activity over time corresponds to dissociation of the A2 domain. The loss of activity profile of wild-type FVIII and FVIII-4A2 was similar but the respective kinetics differed. Indeed, wild-type FVIII had a half-life of 3 min while that of FVIII-4A2 was 11 min. This increased stability may explain the higher specific activity observed in the chromogenic assay. In this test, FVIIIa was incubated for 4 min before adding the substrate. Wild-type FVIII thus lost its activity faster than FVIII-4A2 during this test. The results are shown in FIG. **10**.

Example 8

Construction and Characterization of FVIII-3A2 Mutants

Four triple FVIII-3A2 mutants were constructed: FVIII-3A2 (409-462-507), FVIII-3A2 (462-507-629), FVIII-3A2 (409-462-629), FVIII-3A2 (409-507-629).

FVIII-3A2 (409-462-507) Specific Activity Determination

The specific activity of the FVIII-3A2 mutant (409-462-507) was determined by dividing chromogenic or chromogenic activity by concentration. These specific activities were compared with that of wild-type FVIII. The chromogenic activity of FVIII-3A2 (409-462-507) was 98% of the chromogenic activity of wild-type FVIII. These results indicate that the absence of mutation at position 629 in FVIII-3A2 yielded a higher coagulant activity than for FVIII-4A2.

FVIII-3A2 (409-462-507) Abolition to Inhibition

This mutant was also analyzed for its abolition to inhibition by antibodies from the four hemophiliac patients FS, TD, GC and SL. Residual activity determined after incubation with an inhibitory antibody was divided by the activity remaining after incubation with a non-immune antibody. The percentage of residual activity was thus determined and is presented in FIG. **11** curves. These curves illustrate the residual activity of FVIII-3A2 (409-462-507) after contact with different dilutions of antibodies from the different patients with inhibitors. It clearly appears that the use of the FVIII-3A2 mutant (409-462-507) enable to retain a much higher chronometric activity after incubation with inhibitory antibodies. The combination of mutations 409-462-507 therefore yields a greater abolition to inhibition resulting in an increase in residual activity. This percentage of residual activity depends on both the source of inhibitory antibody and the concentration used.

Example 9

Production of a CHO Cell Line Expressing FVIII-4A2 and Purification/ Production of FVIII

Production of the CHO Cell Line

A CHO cell line (ECACC 85050302) expressing FVIII was generated as described in Plantier et al. (Thrombosis and Haemostasis 2001; 86 p. 596). Briefly, cells were maintained at 37° C. in a humid 5% CO_2 atmosphere. Cells were grown in IMDM medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Cells (7×10^6) were trypsinized and resuspended in PBS, then subjected to electroporation in presence of a cDNA of interest (7 μg). Cells were then reseeded in the presence of geneticin (0.6 mg/ml). Individual clones were selected, subcultured and amplified. Cells' ability to synthesize FVIII was determined by measuring the chromogenic activity of the culture medium. The best producer clones were amplified and grown in triple flasks. Production took place over 5 days during which cells were incubated in complete medium during the day, washed three times, then incubated overnight in IMDM medium containing 1% BSA instead of serum. The BSA-containing medium was collected, centrifuged at 2500 rpm for 10 min at 4° C. and stored at −30° C. Cells were put back into complete medium during the day.

Purification and Production of FVIII Mutants (FVIII-3A2 and FVIII-4A2)

The purification protocol was based on the technique described by Jenkins et al. (Blood, 2004). The culture medium was thawed and 40% (m/V) $(NH_4)_2SO_4$ was added. The medium was shaken overnight at 4° C., then centrifuged at 14,000 rpm for 30 min at 4° C. The pellet was resuspended 1 in 10 by volume in 20 mM MES pH 6.0, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween-20 buffer and dialyzed overnight against a similar buffer but containing 200 mM NaCl. Dialysate was centrifuged at 13,000 rpm for 10 min at room temperature, then loaded at 2 ml/min on a FLPC Sepharose FF column. The column was previously equilibrated with the same buffer. FVIII was eluted in a 0.2 to 1 M NaCl gradient. Fractions containing the highest chromogenic activity were pooled and dialyzed against 50 mM HEPES pH 7.4, 100 mM NaCl, 5 mM NaCl and 0.01% Tween-20 buffer. Dialysate was aliquoted and stored at −80° C. The quality of the protein was assessed after migration on SDS-PAGE 10% acrylamide by silver nitrate staining and by immunoblot. FVIII concentration was determined by the Asserachrom FVIII:Ag kit (Stago, Asnieres, France).

SEQUENCE LISTING

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Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro
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        885                 890                 895

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                935                 940                 945

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            950                 955                 960

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        965                 970                 975

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    980                 985                 990

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1175                1180                1185

agc  aga aac cta ttt ctt  act aac ttg gat aat  tta cat gaa aat      3783
Ser  Arg Asn Leu Phe Leu  Thr Asn Leu Asp Asn  Leu His Glu Asn
1190                1195                1200

aat  aca cac aat caa gaa  aaa aaa att cag gaa  gaa ata gaa aag      3828
Asn  Thr His Asn Gln Glu  Lys Lys Ile Gln Glu  Glu Ile Glu Lys
1205                1210                1215

aag  gaa aca tta atc caa  gag aat gta gtt ttg  cct cag ata cat      3873
Lys  Glu Thr Leu Ile Gln  Glu Asn Val Val Leu  Pro Gln Ile His
1220                1225                1230

aca  gtg act ggc act aag  aat ttc atg aag aac  ctt ttc tta ctg      3918
Thr  Val Thr Gly Thr Lys  Asn Phe Met Lys Asn  Leu Phe Leu Leu
1235                1240                1245

agc  act agg caa aat gta  gaa ggt tca tat gac  ggg gca tat gct      3963
Ser  Thr Arg Gln Asn Val  Glu Gly Ser Tyr Asp  Gly Ala Tyr Ala
1250                1255                1260

cca  gta ctt caa gat ttt  agg tca tta aat gat  tca aca aat aga      4008
Pro  Val Leu Gln Asp Phe  Arg Ser Leu Asn Asp  Ser Thr Asn Arg
1265                1270                1275

aca  aag aaa cac aca gct  cat ttc tca aaa aaa  ggg gag gaa gaa      4053
Thr  Lys Lys His Thr Ala  His Phe Ser Lys Lys  Gly Glu Glu Glu
1280                1285                1290

aac  ttg gaa ggc ttg gga  aat caa acc aag caa  att gta gag aaa      4098
Asn  Leu Glu Gly Leu Gly  Asn Gln Thr Lys Gln  Ile Val Glu Lys
1295                1300                1305

tat  gca tgc acc aca agg  ata tct cct aat aca  agc cag cag aat      4143
Tyr  Ala Cys Thr Thr Arg  Ile Ser Pro Asn Thr  Ser Gln Gln Asn
1310                1315                1320

ttt  gtc acg caa cgt agt  aag aga gct ttg aaa  caa ttc aga ctc      4188
Phe  Val Thr Gln Arg Ser  Lys Arg Ala Leu Lys  Gln Phe Arg Leu
1325                1330                1335

cca  cta gaa gaa aca gaa  ctt gaa aaa agg ata  att gtg gat gac      4233
Pro  Leu Glu Glu Thr Glu  Leu Glu Lys Arg Ile  Ile Val Asp Asp
1340                1345                1350

acc  tca acc cag tgg tcc  aaa aac atg aaa cat  ttg acc ccg agc      4278
Thr  Ser Thr Gln Trp Ser  Lys Asn Met Lys His  Leu Thr Pro Ser
1355                1360                1365

acc  ctc aca cag ata gac  tac aat gag aag gag  aaa ggg gcc att      4323
Thr  Leu Thr Gln Ile Asp  Tyr Asn Glu Lys Glu  Lys Gly Ala Ile
1370                1375                1380

act  cag tct ccc tta tca  gat tgc ctt acg agg  agt cat agc atc      4368
Thr  Gln Ser Pro Leu Ser  Asp Cys Leu Thr Arg  Ser His Ser Ile
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1385                1390                1395

cct  caa gca aat aga tct  cca tta ccc att gca  aag gta tca tca      4413
Pro  Gln Ala Asn Arg Ser  Pro Leu Pro Ile Ala  Lys Val Ser Ser
1400                1405                1410

ttt  cca tct att aga cct  ata tat ctg acc agg  gtc cta ttc caa      4458
Phe  Pro Ser Ile Arg Pro  Ile Tyr Leu Thr Arg  Val Leu Phe Gln
1415                1420                1425

gac  aac tct tct cat ctt  cca gca gca tct tat  aga aag aaa gat      4503
Asp  Asn Ser Ser His Leu  Pro Ala Ala Ser Tyr  Arg Lys Lys Asp
1430                1435                1440

tct  ggg gtc caa gaa agc  agt cat ttc tta caa  gga gcc aaa aaa      4548
Ser  Gly Val Gln Glu Ser  Ser His Phe Leu Gln  Gly Ala Lys Lys
1445                1450                1455

aat  aac ctt tct tta gcc  att cta acc ttg gag  atg act ggt gat      4593
Asn  Asn Leu Ser Leu Ala  Ile Leu Thr Leu Glu  Met Thr Gly Asp
1460                1465                1470

caa  aga gag gtt ggc tcc  ctg ggg aca agt gcc  aca aat tca gtc      4638
Gln  Arg Glu Val Gly Ser  Leu Gly Thr Ser Ala  Thr Asn Ser Val
1475                1480                1485

aca  tac aag aaa gtt gag  aac act gtt ctc ccg  aaa cca gac ttg      4683
Thr  Tyr Lys Lys Val Glu  Asn Thr Val Leu Pro  Lys Pro Asp Leu
1490                1495                1500

ccc  aaa aca tct ggc aaa  gtt gaa ttg ctt cca  aaa gtt cac att      4728
Pro  Lys Thr Ser Gly Lys  Val Glu Leu Leu Pro  Lys Val His Ile
1505                1510                1515

tat  cag aag gac cta ttc  cct acg gaa act agc  aat ggg tct cct      4773
Tyr  Gln Lys Asp Leu Phe  Pro Thr Glu Thr Ser  Asn Gly Ser Pro
1520                1525                1530

ggc  cat ctg gat ctc gtg  gaa ggg agc ctt ctt  cag gga aca gag      4818
Gly  His Leu Asp Leu Val  Glu Gly Ser Leu Leu  Gln Gly Thr Glu
1535                1540                1545

gga  gcg att aag tgg aat  gaa gca aac aga cct  gga aaa gtt ccc      4863
Gly  Ala Ile Lys Trp Asn  Glu Ala Asn Arg Pro  Gly Lys Val Pro
1550                1555                1560

ttt  ctg aga gta gca aca  gaa agc tct gca aag  act ccc tcc aag      4908
Phe  Leu Arg Val Ala Thr  Glu Ser Ser Ala Lys  Thr Pro Ser Lys
1565                1570                1575

cta  ttg gat cct ctt gct  tgg gat aac cac tat  ggt act cag ata      4953
Leu  Leu Asp Pro Leu Ala  Trp Asp Asn His Tyr  Gly Thr Gln Ile
1580                1585                1590

cca  aaa gaa gag tgg aaa  tcc caa gag aag tca  cca gaa aaa aca      4998
Pro  Lys Glu Glu Trp Lys  Ser Gln Glu Lys Ser  Pro Glu Lys Thr
1595                1600                1605

gct  ttt aag aaa aag gat  acc att ttg tcc ctg  aac gct tgt gaa      5043
Ala  Phe Lys Lys Lys Asp  Thr Ile Leu Ser Leu  Asn Ala Cys Glu
1610                1615                1620

agc  aat cat gca ata gca  gca ata aat gag gga  caa aat aag ccc      5088
Ser  Asn His Ala Ile Ala  Ala Ile Asn Glu Gly  Gln Asn Lys Pro
1625                1630                1635

gaa  ata gaa gtc acc tgg  gca aag caa ggt agg  act gaa agg ctg      5133
Glu  Ile Glu Val Thr Trp  Ala Lys Gln Gly Arg  Thr Glu Arg Leu
1640                1645                1650

tgc  tct caa aac cca cca  gtc ttg aaa cgc cat  caa cgg gaa ata      5178
Cys  Ser Gln Asn Pro Pro  Val Leu Lys Arg His  Gln Arg Glu Ile
1655                1660                1665

act  cgt act act ctt cag  tca gat caa gag gaa  att gac tat gat      5223
Thr  Arg Thr Thr Leu Gln  Ser Asp Gln Glu Glu  Ile Asp Tyr Asp
1670                1675                1680

gat  acc ata tca gtt gaa  atg aag aag gaa gat  ttt gac att tat      5268
Asp  Thr Ile Ser Val Glu  Met Lys Lys Glu Asp  Phe Asp Ile Tyr
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1685                1690                1695

gat  gag gat gaa aat cag  agc ccc cgc agc ttt  caa aag aaa aca     5313
Asp  Glu Asp Glu Asn Gln  Ser Pro Arg Ser Phe  Gln Lys Lys Thr
1700                1705                1710

cga  cac tat ttt att gct  gca gtg gag agg ctc  tgg gat tat ggg     5358
Arg  His Tyr Phe Ile Ala  Ala Val Glu Arg Leu  Trp Asp Tyr Gly
1715                1720                1725

atg  agt agc tcc cca cat  gtt cta aga aac agg  gct cag agt ggc     5403
Met  Ser Ser Ser Pro His  Val Leu Arg Asn Arg  Ala Gln Ser Gly
1730                1735                1740

agt  gtc cct cag ttc aag  aaa gtt gtt ttc cag  gaa ttt act gat     5448
Ser  Val Pro Gln Phe Lys  Lys Val Val Phe Gln  Glu Phe Thr Asp
1745                1750                1755

ggc  tcc ttt act cag ccc  tta tac cgt gga gaa  cta aat gaa cat     5493
Gly  Ser Phe Thr Gln Pro  Leu Tyr Arg Gly Glu  Leu Asn Glu His
1760                1765                1770

ttg  gga ctc ctg ggg cca  tat ata aga gca gaa  gtt gaa gat aat     5538
Leu  Gly Leu Leu Gly Pro  Tyr Ile Arg Ala Glu  Val Glu Asp Asn
1775                1780                1785

atc  atg gta act ttc aga  aat cag gcc tct cgt  ccc tat tcc ttc     5583
Ile  Met Val Thr Phe Arg  Asn Gln Ala Ser Arg  Pro Tyr Ser Phe
1790                1795                1800

tat  tct agc ctt att tct  tat gag gaa gat cag  agg caa gga gca     5628
Tyr  Ser Ser Leu Ile Ser  Tyr Glu Glu Asp Gln  Arg Gln Gly Ala
1805                1810                1815

gaa  cct aga aaa aac ttt  gtc aag cct aat gaa  acc aaa act tac     5673
Glu  Pro Arg Lys Asn Phe  Val Lys Pro Asn Glu  Thr Lys Thr Tyr
1820                1825                1830

ttt  tgg aaa gtg caa cat  cat atg gca ccc act  aaa gat gag ttt     5718
Phe  Trp Lys Val Gln His  His Met Ala Pro Thr  Lys Asp Glu Phe
1835                1840                1845

gac  tgc aaa gcc tgg gct  tat ttc tct gat gtt  gac ctg gaa aaa     5763
Asp  Cys Lys Ala Trp Ala  Tyr Phe Ser Asp Val  Asp Leu Glu Lys
1850                1855                1860

gat  gtg cac tca ggc ctg  att gga ccc ctt ctg  gtc tgc cac act     5808
Asp  Val His Ser Gly Leu  Ile Gly Pro Leu Leu  Val Cys His Thr
1865                1870                1875

aac  aca ctg aac cct gct  cat ggg aga caa gtg  aca gta cag gaa     5853
Asn  Thr Leu Asn Pro Ala  His Gly Arg Gln Val  Thr Val Gln Glu
1880                1885                1890

ttt  gct ctg ttt ttc acc  atc ttt gat gag acc  aaa agc tgg tac     5898
Phe  Ala Leu Phe Phe Thr  Ile Phe Asp Glu Thr  Lys Ser Trp Tyr
1895                1900                1905

ttc  act gaa aat atg gaa  aga aac tgc agg gct  ccc tgc aat atc     5943
Phe  Thr Glu Asn Met Glu  Arg Asn Cys Arg Ala  Pro Cys Asn Ile
1910                1915                1920

cag  atg gaa gat ccc act  ttt aaa gag aat tat  cgc ttc cat gca     5988
Gln  Met Glu Asp Pro Thr  Phe Lys Glu Asn Tyr  Arg Phe His Ala
1925                1930                1935

atc  aat ggc tac ata atg  gat aca cta cct ggc  tta gta atg gct     6033
Ile  Asn Gly Tyr Ile Met  Asp Thr Leu Pro Gly  Leu Val Met Ala
1940                1945                1950

cag  gat caa agg att cga  tgg tat ctg ctc agc  atg ggc agc aat     6078
Gln  Asp Gln Arg Ile Arg  Trp Tyr Leu Leu Ser  Met Gly Ser Asn
1955                1960                1965

gaa  aac atc cat tct att  cat ttc agt gga cat  gtg ttc act gta     6123
Glu  Asn Ile His Ser Ile  His Phe Ser Gly His  Val Phe Thr Val
1970                1975                1980

cga  aaa aaa gag gag tat  aaa atg gca ctg tac  aat ctc tat cca     6168
Arg  Lys Lys Glu Glu Tyr  Lys Met Ala Leu Tyr  Asn Leu Tyr Pro
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1985                1990                1995

ggt  gtt ttt gag aca gtg  gaa atg tta cca tcc  aaa gct gga att      6213
Gly  Val Phe Glu Thr Val  Glu Met Leu Pro Ser  Lys Ala Gly Ile
2000                2005                2010

tgg  cgg gtg gaa tgc ctt  att ggc gag cat cta  cat gct ggg atg      6258
Trp  Arg Val Glu Cys Leu  Ile Gly Glu His Leu  His Ala Gly Met
2015                2020                2025

agc  aca ctt ttt ctg gtg  tac agc aat aag tgt  cag act ccc ctg      6303
Ser  Thr Leu Phe Leu Val  Tyr Ser Asn Lys Cys  Gln Thr Pro Leu
2030                2035                2040

gga  atg gct tct gga cac  att aga gat ttt cag  att aca gct tca      6348
Gly  Met Ala Ser Gly His  Ile Arg Asp Phe Gln  Ile Thr Ala Ser
2045                2050                2055

gga  caa tat gga cag tgg  gcc cca aag ctg gcc  aga ctt cat tat      6393
Gly  Gln Tyr Gly Gln Trp  Ala Pro Lys Leu Ala  Arg Leu His Tyr
2060                2065                2070

tcc  gga tca atc aat gcc  tgg agc acc aag gag  ccc ttt tct tgg      6438
Ser  Gly Ser Ile Asn Ala  Trp Ser Thr Lys Glu  Pro Phe Ser Trp
2075                2080                2085

atc  aag gtg gat ctg ttg  gca cca atg att att  cac ggc atc aag      6483
Ile  Lys Val Asp Leu Leu  Ala Pro Met Ile Ile  His Gly Ile Lys
2090                2095                2100

acc  cag ggt gcc cgt cag  aag ttc tcc agc ctc  tac atc tct cag      6528
Thr  Gln Gly Ala Arg Gln  Lys Phe Ser Ser Leu  Tyr Ile Ser Gln
2105                2110                2115

ttt  atc atc atg tat agt  ctt gat ggg aag aag  tgg cag act tat      6573
Phe  Ile Ile Met Tyr Ser  Leu Asp Gly Lys Lys  Trp Gln Thr Tyr
2120                2125                2130

cga  gga aat tcc act gga  acc tta atg gtc ttc  ttt ggc aat gtg      6618
Arg  Gly Asn Ser Thr Gly  Thr Leu Met Val Phe  Phe Gly Asn Val
2135                2140                2145

gat  tca tct ggg ata aaa  cac aat att ttt aac  cct cca att att      6663
Asp  Ser Ser Gly Ile Lys  His Asn Ile Phe Asn  Pro Pro Ile Ile
2150                2155                2160

gct  cga tac atc cgt ttg  cac cca act cat tat  agc att cgc agc      6708
Ala  Arg Tyr Ile Arg Leu  His Pro Thr His Tyr  Ser Ile Arg Ser
2165                2170                2175

act  ctt cgc atg gag ttg  atg ggc tgt gat tta  aat agt tgc agc      6753
Thr  Leu Arg Met Glu Leu  Met Gly Cys Asp Leu  Asn Ser Cys Ser
2180                2185                2190

atg  cca ttg gga atg gag  agt aaa gca ata tca  gat gca cag att      6798
Met  Pro Leu Gly Met Glu  Ser Lys Ala Ile Ser  Asp Ala Gln Ile
2195                2200                2205

act  gct tca tcc tac ttt  acc aat atg ttt gcc  acc tgg tct cct      6843
Thr  Ala Ser Ser Tyr Phe  Thr Asn Met Phe Ala  Thr Trp Ser Pro
2210                2215                2220

tca  aaa gct cga ctt cac  ctc caa ggg agg agt  aat gcc tgg aga      6888
Ser  Lys Ala Arg Leu His  Leu Gln Gly Arg Ser  Asn Ala Trp Arg
2225                2230                2235

cct  cag gtg aat aat cca  aaa gag tgg ctg caa  gtg gac ttc cag      6933
Pro  Gln Val Asn Asn Pro  Lys Glu Trp Leu Gln  Val Asp Phe Gln
2240                2245                2250

aag  aca atg aaa gtc aca  gga gta act act cag  gga gta aaa tct      6978
Lys  Thr Met Lys Val Thr  Gly Val Thr Thr Gln  Gly Val Lys Ser
2255                2260                2265

ctg  ctt acc agc atg tat  gtg aag gag ttc ctc  atc tcc agc agt      7023
Leu  Leu Thr Ser Met Tyr  Val Lys Glu Phe Leu  Ile Ser Ser Ser
2270                2275                2280

caa  gat ggc cat cag tgg  act ctc ttt ttt cag  aat ggc aaa gta      7068
Gln  Asp Gly His Gln Trp  Thr Leu Phe Phe Gln  Asn Gly Lys Val
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2285                2290                2295

aag  gtt ttt cag gga aat  caa gac tcc ttc aca  cct gtg gtg aac      7113
Lys  Val Phe Gln Gly Asn  Gln Asp Ser Phe Thr  Pro Val Val Asn
2300                2305                2310

tct  cta gac cca ccg tta  ctg act cgc tac ctt  cga att cac ccc      7158
Ser  Leu Asp Pro Pro Leu  Leu Thr Arg Tyr Leu  Arg Ile His Pro
2315                2320                2325

cag  agt tgg gtg cac cag  att gcc ctg agg atg  gag gtt ctg ggc      7203
Gln  Ser Trp Val His Gln  Ile Ala Leu Arg Met  Glu Val Leu Gly
2330                2335                2340

tgc  gag gca cag gac ctc  tac tga gggtggccac tgcagcacct            7247
Cys  Glu Ala Gln Asp Leu  Tyr
2345                2350

gccactgccg tcacctctcc ctcctcagct ccagggcagt gtccctccct ggcttgcctt   7307

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aatgatgcag gtgcaaatgg tttatagccc tgtgaagttc ttaaagttta gaggctaact   8807

tacagaaatg aataagttgt tttgttttat agcccggtag aggagttaac cccaaaggtg   8867

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<211> LENGTH: 2351
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser
            20                  25                  30

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
        35                  40                  45

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
    50                  55                  60

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
65                  70                  75                  80

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
                85                  90                  95

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
            100                 105                 110

His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
        115                 120                 125

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
    130                 135                 140

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
145                 150                 155                 160

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
                165                 170                 175

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
            180                 185                 190

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
        195                 200                 205

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
    210                 215                 220

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
225                 230                 235                 240

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
                245                 250                 255

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
            260                 265                 270

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
        275                 280                 285

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
    290                 295                 300

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
305                 310                 315                 320

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
                325                 330                 335

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
            340                 345                 350

Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
        355                 360                 365

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
    370                 375                 380

Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
385                 390                 395                 400
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Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro
                405                 410                 415

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
            420                 425                 430

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
        435                 440                 445

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
    450                 455                 460

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
465                 470                 475                 480

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
                485                 490                 495

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
            500                 505                 510

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
        515                 520                 525

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
    530                 535                 540

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
545                 550                 555                 560

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
                565                 570                 575

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
            580                 585                 590

Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
        595                 600                 605

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
    610                 615                 620

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
625                 630                 635                 640

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
                645                 650                 655

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
            660                 665                 670

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
        675                 680                 685

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
    690                 695                 700

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
705                 710                 715                 720

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
                725                 730                 735

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
            740                 745                 750

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro
        755                 760                 765

Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp
    770                 775                 780

Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys
785                 790                 795                 800

Ile Gln Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser
                805                 810                 815

Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr
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            820                 825                 830

Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn
        835                 840                 845

Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly
    850                 855                 860

Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu
865                 870                 875                 880

Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys
                885                 890                 895

Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn
            900                 905                 910

Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met
        915                 920                 925

Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys
    930                 935                 940

Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu
945                 950                 955                 960

Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu
                965                 970                 975

Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
            980                 985                 990

Lys Gly Lys Arg Ala His Gly Pro  Ala Leu Leu Thr Lys  Asp Asn Ala
        995                 1000                1005

Leu Phe  Lys Val Ser Ile Ser  Leu Leu Lys Thr Asn  Lys Thr Ser
    1010                1015                1020

Asn Asn  Ser Ala Thr Asn Arg  Lys Thr His Ile Asp  Gly Pro Ser
    1025                1030                1035

Leu Leu  Ile Glu Asn Ser Pro  Ser Val Trp Gln Asn  Ile Leu Glu
    1040                1045                1050

Ser Asp  Thr Glu Phe Lys Lys  Val Thr Pro Leu Ile  His Asp Arg
    1055                1060                1065

Met Leu  Met Asp Lys Asn Ala  Thr Ala Leu Arg Leu  Asn His Met
    1070                1075                1080

Ser Asn  Lys Thr Thr Ser Ser  Lys Asn Met Glu Met  Val Gln Gln
    1085                1090                1095

Lys Lys  Glu Gly Pro Ile Pro  Pro Asp Ala Gln Asn  Pro Asp Met
    1100                1105                1110

Ser Phe  Phe Lys Met Leu Phe  Leu Pro Glu Ser Ala  Arg Trp Ile
    1115                1120                1125

Gln Arg  Thr His Gly Lys Asn  Ser Leu Asn Ser Gly  Gln Gly Pro
    1130                1135                1140

Ser Pro  Lys Gln Leu Val Ser  Leu Gly Pro Glu Lys  Ser Val Glu
    1145                1150                1155

Gly Gln  Asn Phe Leu Ser Glu  Lys Asn Lys Val Val  Val Gly Lys
    1160                1165                1170

Gly Glu  Phe Thr Lys Asp Val  Gly Leu Lys Glu Met  Val Phe Pro
    1175                1180                1185

Ser Ser  Arg Asn Leu Phe Leu  Thr Asn Leu Asp Asn  Leu His Glu
    1190                1195                1200

Asn Asn  Thr His Asn Gln Glu  Lys Lys Ile Gln Glu  Glu Ile Glu
    1205                1210                1215

Lys Lys  Glu Thr Leu Ile Gln  Glu Asn Val Val Leu  Pro Gln Ile
    1220                1225                1230
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His Thr  Val Thr Gly Thr Lys  Asn Phe Met Lys Asn  Leu Phe Leu
    1235                 1240                 1245

Leu Ser  Thr Arg Gln Asn Val  Glu Gly Ser Tyr Asp  Gly Ala Tyr
    1250                 1255                 1260

Ala Pro  Val Leu Gln Asp Phe  Arg Ser Leu Asn Asp  Ser Thr Asn
    1265                 1270                 1275

Arg Thr  Lys Lys His Thr Ala  His Phe Ser Lys Lys  Gly Glu Glu
    1280                 1285                 1290

Glu Asn  Leu Glu Gly Leu Gly  Asn Gln Thr Lys Gln  Ile Val Glu
    1295                 1300                 1305

Lys Tyr  Ala Cys Thr Thr Arg  Ile Ser Pro Asn Thr  Ser Gln Gln
    1310                 1315                 1320

Asn Phe  Val Thr Gln Arg Ser  Lys Arg Ala Leu Lys  Gln Phe Arg
    1325                 1330                 1335

Leu Pro  Leu Glu Glu Thr Glu  Leu Glu Lys Arg Ile  Ile Val Asp
    1340                 1345                 1350

Asp Thr  Ser Thr Gln Trp Ser  Lys Asn Met Lys His  Leu Thr Pro
    1355                 1360                 1365

Ser Thr  Leu Thr Gln Ile Asp  Tyr Asn Glu Lys Glu  Lys Gly Ala
    1370                 1375                 1380

Ile Thr  Gln Ser Pro Leu Ser  Asp Cys Leu Thr Arg  Ser His Ser
    1385                 1390                 1395

Ile Pro  Gln Ala Asn Arg Ser  Pro Leu Pro Ile Ala  Lys Val Ser
    1400                 1405                 1410

Ser Phe  Pro Ser Ile Arg Pro  Ile Tyr Leu Thr Arg  Val Leu Phe
    1415                 1420                 1425

Gln Asp  Asn Ser Ser His Leu  Pro Ala Ala Ser Tyr  Arg Lys Lys
    1430                 1435                 1440

Asp Ser  Gly Val Gln Glu Ser  Ser His Phe Leu Gln  Gly Ala Lys
    1445                 1450                 1455

Lys Asn  Asn Leu Ser Leu Ala  Ile Leu Thr Leu Glu  Met Thr Gly
    1460                 1465                 1470

Asp Gln  Arg Glu Val Gly Ser  Leu Gly Thr Ser Ala  Thr Asn Ser
    1475                 1480                 1485

Val Thr  Tyr Lys Lys Val Glu  Asn Thr Val Leu Pro  Lys Pro Asp
    1490                 1495                 1500

Leu Pro  Lys Thr Ser Gly Lys  Val Glu Leu Leu Pro  Lys Val His
    1505                 1510                 1515

Ile Tyr  Gln Lys Asp Leu Phe  Pro Thr Glu Thr Ser  Asn Gly Ser
    1520                 1525                 1530

Pro Gly  His Leu Asp Leu Val  Glu Gly Ser Leu Leu  Gln Gly Thr
    1535                 1540                 1545

Glu Gly  Ala Ile Lys Trp Asn  Glu Ala Asn Arg Pro  Gly Lys Val
    1550                 1555                 1560

Pro Phe  Leu Arg Val Ala Thr  Glu Ser Ser Ala Lys  Thr Pro Ser
    1565                 1570                 1575

Lys Leu  Leu Asp Pro Leu Ala  Trp Asp Asn His Tyr  Gly Thr Gln
    1580                 1585                 1590

Ile Pro  Lys Glu Glu Trp Lys  Ser Gln Glu Lys Ser  Pro Glu Lys
    1595                 1600                 1605

Thr Ala  Phe Lys Lys Lys Asp  Thr Ile Leu Ser Leu  Asn Ala Cys
    1610                 1615                 1620

Glu Ser  Asn His Ala Ile Ala  Ala Ile Asn Glu Gly  Gln Asn Lys
    1625                 1630                 1635
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Pro Glu  Ile Glu Val Thr Trp  Ala Lys Gln Gly Arg  Thr Glu Arg
    1640                 1645                 1650

Leu Cys  Ser Gln Asn Pro Pro  Val Leu Lys Arg His  Gln Arg Glu
    1655                 1660                 1665

Ile Thr  Arg Thr Thr Leu Gln  Ser Asp Gln Glu Glu  Ile Asp Tyr
    1670                 1675                 1680

Asp Asp  Thr Ile Ser Val Glu  Met Lys Lys Glu Asp  Phe Asp Ile
    1685                 1690                 1695

Tyr Asp  Glu Asp Glu Asn Gln  Ser Pro Arg Ser Phe  Gln Lys Lys
    1700                 1705                 1710

Thr Arg  His Tyr Phe Ile Ala  Ala Val Glu Arg Leu  Trp Asp Tyr
    1715                 1720                 1725

Gly Met  Ser Ser Ser Pro His  Val Leu Arg Asn Arg  Ala Gln Ser
    1730                 1735                 1740

Gly Ser  Val Pro Gln Phe Lys  Lys Val Val Phe Gln  Glu Phe Thr
    1745                 1750                 1755

Asp Gly  Ser Phe Thr Gln Pro  Leu Tyr Arg Gly Glu  Leu Asn Glu
    1760                 1765                 1770

His Leu  Gly Leu Leu Gly Pro  Tyr Ile Arg Ala Glu  Val Glu Asp
    1775                 1780                 1785

Asn Ile  Met Val Thr Phe Arg  Asn Gln Ala Ser Arg  Pro Tyr Ser
    1790                 1795                 1800

Phe Tyr  Ser Ser Leu Ile Ser  Tyr Glu Glu Asp Gln  Arg Gln Gly
    1805                 1810                 1815

Ala Glu  Pro Arg Lys Asn Phe  Val Lys Pro Asn Glu  Thr Lys Thr
    1820                 1825                 1830

Tyr Phe  Trp Lys Val Gln His  His Met Ala Pro Thr  Lys Asp Glu
    1835                 1840                 1845

Phe Asp  Cys Lys Ala Trp Ala  Tyr Phe Ser Asp Val  Asp Leu Glu
    1850                 1855                 1860

Lys Asp  Val His Ser Gly Leu  Ile Gly Pro Leu Leu  Val Cys His
    1865                 1870                 1875

Thr Asn  Thr Leu Asn Pro Ala  His Gly Arg Gln Val  Thr Val Gln
    1880                 1885                 1890

Glu Phe  Ala Leu Phe Phe Thr  Ile Phe Asp Glu Thr  Lys Ser Trp
    1895                 1900                 1905

Tyr Phe  Thr Glu Asn Met Glu  Arg Asn Cys Arg Ala  Pro Cys Asn
    1910                 1915                 1920

Ile Gln  Met Glu Asp Pro Thr  Phe Lys Glu Asn Tyr  Arg Phe His
    1925                 1930                 1935

Ala Ile  Asn Gly Tyr Ile Met  Asp Thr Leu Pro Gly  Leu Val Met
    1940                 1945                 1950

Ala Gln  Asp Gln Arg Ile Arg  Trp Tyr Leu Leu Ser  Met Gly Ser
    1955                 1960                 1965

Asn Glu  Asn Ile His Ser Ile  His Phe Ser Gly His  Val Phe Thr
    1970                 1975                 1980

Val Arg  Lys Lys Glu Glu Tyr  Lys Met Ala Leu Tyr  Asn Leu Tyr
    1985                 1990                 1995

Pro Gly  Val Phe Glu Thr Val  Glu Met Leu Pro Ser  Lys Ala Gly
    2000                 2005                 2010

Ile Trp  Arg Val Glu Cys Leu  Ile Gly Glu His Leu  His Ala Gly
    2015                 2020                 2025

Met Ser  Thr Leu Phe Leu Val  Tyr Ser Asn Lys Cys  Gln Thr Pro
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    2030                2035                2040

Leu Gly  Met Ala Ser Gly His  Ile Arg Asp Phe Gln  Ile Thr Ala
    2045                2050                2055

Ser Gly  Gln Tyr Gly Gln Trp  Ala Pro Lys Leu Ala  Arg Leu His
    2060                2065                2070

Tyr Ser  Gly Ser Ile Asn Ala  Trp Ser Thr Lys Glu  Pro Phe Ser
    2075                2080                2085

Trp Ile  Lys Val Asp Leu Leu  Ala Pro Met Ile Ile  His Gly Ile
    2090                2095                2100

Lys Thr  Gln Gly Ala Arg Gln  Lys Phe Ser Ser Leu  Tyr Ile Ser
    2105                2110                2115

Gln Phe  Ile Ile Met Tyr Ser  Leu Asp Gly Lys Lys  Trp Gln Thr
    2120                2125                2130

Tyr Arg  Gly Asn Ser Thr Gly  Thr Leu Met Val Phe  Phe Gly Asn
    2135                2140                2145

Val Asp  Ser Ser Gly Ile Lys  His Asn Ile Phe Asn  Pro Pro Ile
    2150                2155                2160

Ile Ala  Arg Tyr Ile Arg Leu  His Pro Thr His Tyr  Ser Ile Arg
    2165                2170                2175

Ser Thr  Leu Arg Met Glu Leu  Met Gly Cys Asp Leu  Asn Ser Cys
    2180                2185                2190

Ser Met  Pro Leu Gly Met Glu  Ser Lys Ala Ile Ser  Asp Ala Gln
    2195                2200                2205

Ile Thr  Ala Ser Ser Tyr Phe  Thr Asn Met Phe Ala  Thr Trp Ser
    2210                2215                2220

Pro Ser  Lys Ala Arg Leu His  Leu Gln Gly Arg Ser  Asn Ala Trp
    2225                2230                2235

Arg Pro  Gln Val Asn Asn Pro  Lys Glu Trp Leu Gln  Val Asp Phe
    2240                2245                2250

Gln Lys  Thr Met Lys Val Thr  Gly Val Thr Thr Gln  Gly Val Lys
    2255                2260                2265

Ser Leu  Leu Thr Ser Met Tyr  Val Lys Glu Phe Leu  Ile Ser Ser
    2270                2275                2280

Ser Gln  Asp Gly His Gln Trp  Thr Leu Phe Phe Gln  Asn Gly Lys
    2285                2290                2295

Val Lys  Val Phe Gln Gly Asn  Gln Asp Ser Phe Thr  Pro Val Val
    2300                2305                2310

Asn Ser  Leu Asp Pro Pro Leu  Leu Thr Arg Tyr Leu  Arg Ile His
    2315                2320                2325

Pro Gln  Ser Trp Val His Gln  Ile Ala Leu Arg Met  Glu Val Leu
    2330                2335                2340

Gly Cys  Glu Ala Gln Asp Leu  Tyr
    2345                2350


<210> SEQ ID NO 3
<211> LENGTH: 2332
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1               5                   10                  15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
            20                  25                  30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
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        35                  40                  45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
    50                  55                  60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65                  70                  75                  80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85                  90                  95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130                 135                 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                165                 170                 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180                 185                 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195                 200                 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210                 215                 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                245                 250                 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275                 280                 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                325                 330                 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
            340                 345                 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370                 375                 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460
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Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
            740                 745                 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
                805                 810                 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
        835                 840                 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850                 855                 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865                 870                 875                 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
                885                 890                 895
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Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
        915                 920                 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930                 935                 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
                965                 970                 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990

Val Ser Ile Ser Leu Leu Lys Thr  Asn Lys Thr Ser Asn  Asn Ser Ala
        995                 1000                1005

Thr Asn  Arg Lys Thr His Ile  Asp Gly Pro Ser Leu  Leu Ile Glu
    1010                1015                1020

Asn Ser  Pro Ser Val Trp Gln  Asn Ile Leu Glu Ser  Asp Thr Glu
    1025                1030                1035

Phe Lys  Lys Val Thr Pro Leu  Ile His Asp Arg Met  Leu Met Asp
    1040                1045                1050

Lys Asn  Ala Thr Ala Leu Arg  Leu Asn His Met Ser  Asn Lys Thr
    1055                1060                1065

Thr Ser  Ser Lys Asn Met Glu  Met Val Gln Gln Lys  Lys Glu Gly
    1070                1075                1080

Pro Ile  Pro Pro Asp Ala Gln  Asn Pro Asp Met Ser  Phe Phe Lys
    1085                1090                1095

Met Leu  Phe Leu Pro Glu Ser  Ala Arg Trp Ile Gln  Arg Thr His
    1100                1105                1110

Gly Lys  Asn Ser Leu Asn Ser  Gly Gln Gly Pro Ser  Pro Lys Gln
    1115                1120                1125

Leu Val  Ser Leu Gly Pro Glu  Lys Ser Val Glu Gly  Gln Asn Phe
    1130                1135                1140

Leu Ser  Glu Lys Asn Lys Val  Val Val Gly Lys Gly  Glu Phe Thr
    1145                1150                1155

Lys Asp  Val Gly Leu Lys Glu  Met Val Phe Pro Ser  Ser Arg Asn
    1160                1165                1170

Leu Phe  Leu Thr Asn Leu Asp  Asn Leu His Glu Asn  Asn Thr His
    1175                1180                1185

Asn Gln  Glu Lys Lys Ile Gln  Glu Glu Ile Glu Lys  Lys Glu Thr
    1190                1195                1200

Leu Ile  Gln Glu Asn Val Val  Leu Pro Gln Ile His  Thr Val Thr
    1205                1210                1215

Gly Thr  Lys Asn Phe Met Lys  Asn Leu Phe Leu Leu  Ser Thr Arg
    1220                1225                1230

Gln Asn  Val Glu Gly Ser Tyr  Asp Gly Ala Tyr Ala  Pro Val Leu
    1235                1240                1245

Gln Asp  Phe Arg Ser Leu Asn  Asp Ser Thr Asn Arg  Thr Lys Lys
    1250                1255                1260

His Thr  Ala His Phe Ser Lys  Lys Gly Glu Glu Glu  Asn Leu Glu
    1265                1270                1275

Gly Leu  Gly Asn Gln Thr Lys  Gln Ile Val Glu Lys  Tyr Ala Cys
    1280                1285                1290

Thr Thr  Arg Ile Ser Pro Asn  Thr Ser Gln Gln Asn  Phe Val Thr
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    1295                1300                1305

Gln Arg  Ser Lys Arg Ala Leu  Lys Gln Phe Arg Leu  Pro Leu Glu
    1310                1315                1320

Glu Thr  Glu Leu Glu Lys Arg  Ile Ile Val Asp Asp  Thr Ser Thr
    1325                1330                1335

Gln Trp  Ser Lys Asn Met Lys  His Leu Thr Pro Ser  Thr Leu Thr
    1340                1345                1350

Gln Ile  Asp Tyr Asn Glu Lys  Glu Lys Gly Ala Ile  Thr Gln Ser
    1355                1360                1365

Pro Leu  Ser Asp Cys Leu Thr  Arg Ser His Ser Ile  Pro Gln Ala
    1370                1375                1380

Asn Arg  Ser Pro Leu Pro Ile  Ala Lys Val Ser Ser  Phe Pro Ser
    1385                1390                1395

Ile Arg  Pro Ile Tyr Leu Thr  Arg Val Leu Phe Gln  Asp Asn Ser
    1400                1405                1410

Ser His  Leu Pro Ala Ala Ser  Tyr Arg Lys Lys Asp  Ser Gly Val
    1415                1420                1425

Gln Glu  Ser Ser His Phe Leu  Gln Gly Ala Lys Lys  Asn Asn Leu
    1430                1435                1440

Ser Leu  Ala Ile Leu Thr Leu  Glu Met Thr Gly Asp  Gln Arg Glu
    1445                1450                1455

Val Gly  Ser Leu Gly Thr Ser  Ala Thr Asn Ser Val  Thr Tyr Lys
    1460                1465                1470

Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
    1475                1480                1485

Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys
    1490                1495                1500

Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
    1505                1510                1515

Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
    1520                1525                1530

Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
    1535                1540                1545

Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
    1550                1555                1560

Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
    1565                1570                1575

Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
    1580                1585                1590

Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
    1595                1600                1605

Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
    1610                1615                1620

Val Thr  Trp Ala Lys Gln Gly  Arg Thr Glu Arg Leu  Cys Ser Gln
    1625                1630                1635

Asn Pro  Pro Val Leu Lys Arg  His Gln Arg Glu Ile  Thr Arg Thr
    1640                1645                1650

Thr Leu  Gln Ser Asp Gln Glu  Glu Ile Asp Tyr Asp  Asp Thr Ile
    1655                1660                1665

Ser Val  Glu Met Lys Lys Glu  Asp Phe Asp Ile Tyr  Asp Glu Asp
    1670                1675                1680

Glu Asn  Gln Ser Pro Arg Ser  Phe Gln Lys Lys Thr  Arg His Tyr
    1685                1690                1695
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Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser
    1700                1705                1710

Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
    1715                1720                1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe
    1730                1735                1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu
    1745                1750                1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
    1760                1765                1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
    1775                1780                1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
    1790                1795                1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys
    1805                1810                1815

Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
    1820                1825                1830

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
    1835                1840                1845

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu
    1850                1855                1860

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu
    1865                1870                1875

Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu
    1880                1885                1890

Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu
    1895                1900                1905

Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly
    1910                1915                1920

Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln
    1925                1930                1935

Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile
    1940                1945                1950

His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
    1955                1960                1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe
    1970                1975                1980

Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val
    1985                1990                1995

Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu
    2000                2005                2010

Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala
    2015                2020                2025

Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr
    2030                2035                2040

Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser
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Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val
    2060                2065                2070

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly
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Gly Ile  Lys His Asn Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr
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Ile Arg  Leu His Pro Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg
    2150                 2155                 2160

Met Glu  Leu Met Gly Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu
    2165                 2170                 2175

Gly Met  Glu Ser Lys Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser
    2180                 2185                 2190

Ser Tyr  Phe Thr Asn Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala
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Arg Leu  His Leu Gln Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val
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Asn Asn  Pro Lys Glu Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met
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Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
    2240                 2245                 2250

Ser Met  Tyr Val Lys Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly
    2255                 2260                 2265

His Gln  Trp Thr Leu Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe
    2270                 2275                 2280

Gln Gly  Asn Gln Asp Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp
    2285                 2290                 2295

Pro Pro  Leu Leu Thr Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp
    2300                 2305                 2310

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<223> OTHER INFORMATION: FVIII without domain B

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Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
    50                  55                  60

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
65                  70                  75                  80

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
                85                  90                  95

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
            100                 105                 110

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
        115                 120                 125

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
    130                 135                 140

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
145                 150                 155                 160

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
                165                 170                 175

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
            180                 185                 190

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
        195                 200                 205

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
    210                 215                 220

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
225                 230                 235                 240

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
                245                 250                 255

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
            260                 265                 270

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
        275                 280                 285

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
    290                 295                 300

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
305                 310                 315                 320

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
                325                 330                 335

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
            340                 345                 350

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
        355                 360                 365

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
    370                 375                 380

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
385                 390                 395                 400

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
                405                 410                 415

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
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Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
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Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
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Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
465                 470                 475                 480

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
                485                 490                 495

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
            500                 505                 510

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
        515                 520                 525

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
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Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
545                 550                 555                 560

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
                565                 570                 575

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
            580                 585                 590

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
        595                 600                 605

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
    610                 615                 620

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
625                 630                 635                 640

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
                645                 650                 655

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
            660                 665                 670

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
        675                 680                 685

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
    690                 695                 700

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
705                 710                 715                 720

Ile Glu Pro Arg Arg Arg Arg Glu Ile Thr Arg Thr Thr Leu Gln
                725                 730                 735

Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met
            740                 745                 750

Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro
        755                 760                 765

Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu
    770                 775                 780

Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn
785                 790                 795                 800

Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln
                805                 810                 815

Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu
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Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu
        835                 840                 845

Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
    850                 855                 860

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala
```

```
865                 870                 875                 880

Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe
                885                 890                 895

Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys
            900                 905                 910

Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
        915                 920                 925

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn
    930                 935                 940

Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe
945                 950                 955                 960

Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu
                965                 970                 975

Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe
            980                 985                 990

Lys Glu Asn Tyr Arg Phe His Ala  Ile Asn Gly Tyr Ile  Met Asp Thr
        995                 1000                1005

Leu Pro  Gly Leu Val Met Ala  Gln Asp Gln Arg Ile  Arg Trp Tyr
    1010                1015                1020

Leu Leu  Ser Met Gly Ser Asn  Glu Asn Ile His Ser  Ile His Phe
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Ser Gly  His Val Phe Thr Val  Arg Lys Lys Glu Glu  Tyr Lys Met
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Ala Leu  Tyr Asn Leu Tyr Pro  Gly Val Phe Glu Thr  Val Glu Met
    1055                1060                1065

Leu Pro  Ser Lys Ala Gly Ile  Trp Arg Val Glu Cys  Leu Ile Gly
    1070                1075                1080

Glu His  Leu His Ala Gly Met  Ser Thr Leu Phe Leu  Val Tyr Ser
    1085                1090                1095

Asn Lys  Cys Gln Thr Pro Leu  Gly Met Ala Ser Gly  His Ile Arg
    1100                1105                1110

Asp Phe  Gln Ile Thr Ala Ser  Gly Gln Tyr Gly Gln  Trp Ala Pro
    1115                1120                1125

Lys Leu  Ala Arg Leu His Tyr  Ser Gly Ser Ile Asn  Ala Trp Ser
    1130                1135                1140

Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val Asp Leu  Leu Ala Pro
    1145                1150                1155

Met Ile  Ile His Gly Ile Lys  Thr Gln Gly Ala Arg  Gln Lys Phe
    1160                1165                1170

Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile Met Tyr  Ser Leu Asp
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Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn Ser Thr  Gly Thr Leu
    1190                1195                1200

Met Val  Phe Phe Gly Asn Val  Asp Ser Ser Gly Ile  Lys His Asn
    1205                1210                1215

Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr Ile Arg  Leu His Pro
    1220                1225                1230

Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg Met Glu  Leu Met Gly
    1235                1240                1245

Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu Gly Met  Glu Ser Lys
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Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser Ser Tyr  Phe Thr Asn
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	1295					1300					1305			
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Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	Gly	His	Gln	Trp	Thr	Leu
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Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe	Gln	Gly	Asn	Gln	Asp
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Ser	Phe	Thr	Pro	Val	Val	Asn	Ser	Leu	Asp	Pro	Pro	Leu	Leu	Thr
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Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp	Val	His	Gln	Ile	Ala
	1385					1390					1395			
Leu	Arg	Met	Glu	Val	Leu	Gly	Cys	Glu	Ala	Gln	Asp	Leu	Tyr	
	1400					1405					1410			

The invention claimed is:

1. An isolated human coagulation factor VIII (FVIII) variant comprising a substitution of the amino acid at position 462 of SEQ ID NO: 3, wherein said variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII, retains procoagulant activity and, optionally, totally or partially lacks the domain B, and wherein the polypeptide sequence of the variant differs from SEQ ID NO: 3 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 substitutions, without including the optional total or partial deletion of the domain B.

2. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant comprises a single amino acid substitution.

3. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant further comprises a substitution of at least one amino acid selected from the group consisting of the amino acids at position 2202 and 437 of SEQ ID NO: 3.

4. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant contains a combination of two substitutions selected from the group consisting of the amino acids at positions 409+462, 462+507 and, 462+629 of SEQ ID NO: 3.

5. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant contains a combination of three substitutions selected from the group consisting of the amino acids at positions 409+462+507, 462+507+629, and 409+462+629 of SEQ ID NO: 3.

6. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant contains a combination of four substitutions of the amino acids at positions 409, 462, 507 and 629 of SEQ ID NO: 3.

7. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant further comprises a substitution of at least one amino acid selected from the group consisting of the amino acids at positions 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of SEQ ID NO: 3.

8. The isolated human coagulation FVIII variant according to claim **1**, wherein said variant further comprises a substitution of at least one amino acid selected from the group consisting of the amino acids at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of SEQ ID NO: 3.

9. The isolated human coagulation FVIII variant according to claim **1**, wherein the amino acid is substituted by an amino acid selected from an Alanine, a Methionine, a Serine, or a Glycine.

10. The isolated human coagulation FVIII variant according to claim **9**, wherein the substituted amino acid is an Alanine.

11. The isolated human coagulation FVIII variant according to claim **1**, said human coagulation FVIII variant comprising a substitution of the amino acid at position 462 of SEQ ID NO: 3 and said substitution of 1 to 15 amino acids at a position in SEQ ID NO: 3 is selected from the group consisting of 400, 403, 409, 414, 421, 437, 486, 493, 494, 496, 507, 518, 562, 629, 2175, 2177, 2183, 2186, 2191, 2196, 2199, 2200, 2202, 2204, 2205, 2206, 2212, 2213, 2215, 2217, 2226, 2235, 2244, 2251, 2252, 2258, 2261, 2264, 2268, 2269, 2275, 2278, 2280, 2281, 2282, 2289, 2294, 2311, 2312 and 2316, wherein said variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII, retains procoagulant activity and, optionally, totally or partially lacks the domain B.

12. A pharmaceutical composition comprising the isolated human coagulation FVIII variant according to claim **1** and a pharmaceutically acceptable carrier or excipient.

13. A method for treating hemophilia A in a patient, comprising administering the isolated human coagulation FVIII variant according to claim **1** to said patient.

14. The method according to claim **13**, wherein the patient to be treated is a hemophiliac patient with inhibitors.

15. The method according to claim **13**, wherein the patient to be treated is a hemophiliac patient before the development of inhibitors.

16. A method for determining an inhibitor type in a patient with hemophilia A comprising performing a recognition test of inhibitory antibodies contained in a serum sample from the patient on one or more isolated human coagulation FVIII variants according to claim **1**.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,623,824 B2
APPLICATION NO. : 12/528379
DATED : January 7, 2014
INVENTOR(S) : Didier Saboulard et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Column 3,
Line 2, “FVIIII variants” should read --FVIII variants--.
Line 8, “US20021165177;” should read --US2002/165177;--.

Column 4,
Line 60, “FVIIII activity” should read --FVIII activity--.

Column 5,
Line 10, “antibodies not” should read --antibodies do not--.

Column 6,
Line 61, “group speculate” should read --group speculates--.

Column 7,
Line 49, “five six,” should read --five, six,--.

Column 10,
Line 5, “(TD, GC, PR, SL and FS)” should read --(TD (Figure 6C), GC (Figure 6B), PR (Figure 6D), SL (Figure 6E) and FS (Figure 6A))--.
Lines 11-12, “(GMA012) and a rabbit polyclonal antibody.” should read --(GMA012, Figure 7A) and a rabbit polyclonal antibody (Figure 7B).--.
Lines 15-16, “(ESH4) and anti-A2 domain antibody (GMA012).” should read --(ESH4, Figure 8B) and anti-A2 domain antibody (GMA012, Figure 8A).--.
Lines 24-25, “(TD, GC, SL and FS measured by Bethesda assay.
FIGS. **12-14**: Primary screen results; list of **158** Alanine”

should read

Signed and Sealed this
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--(TD (Figure 11A), GC (Figure 11B), SL (Figure 11D) and FS (Figure 11C)) measured by Bethesda assay.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

FIGS. **12-14**: Primary screen results; list of 158 Alanine--.

Column 10, Line 42 through Column 14, Line 47,

"**FIG. 21**: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

Description of the invention

The present invention provides a solution to resolve a serious complication that occurs in 30% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an optional relative loss of activity in variants whose mutations confer a abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate a optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: **5409A** indicates the substitution of the serine residue at position **409** of SEQ ID No. 3 by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non-naturally occuring amino acid. The terms "substitution" and "mutation" are interchangeable. The sign "+" indicates a combination of substitutions.

"Comprise" means that the variant or the fragment thereof has one or more substitutions such as indicated with reference to SEQ ID No. 3, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the **158** selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier's instructions. Briefly, culture supernatants (50 µl) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50 µl), preheated at 37° C., was then added for 4 min, after which 50 µl of development medium at 37° C. were added. The formation of product over time was

Column 10, Line 42 through Column 14, Line 47 cont'd.

measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

FIGS. **12-14** show the activities of the **158** mutants which retained more than 50% of non-mutated FVIII activity. Said **158** mutants were selected for the secondary screening.

Example **4**: Secondary screen: Evaluation of loss of antigenicity towards human FVIII inhibitory antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the **158** mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chronometric assay.

Culture supernatants obtained after **48** h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chronometric activity comprised in the range of about 10-20% (1 FVIII unit = 100% activity = 200 ng/ml). The culture supernatant diluted or not (140 μ1) was added to 150 μl of FVIII-depleted human plasma (Stago, Asnieres, France). An antibody dilution (10 μl) was then added to the mix. These antibodies are IgG fractions purified on protein A- from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained.

Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1h30. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-l'Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient's serum. Said percentage was calculated as shown in Figure **5** for the FVIII mutant E**518**A. Abolition to inhibition expressed is a percentage =-[(b-a)/a] × 100; where "a" is the percentage residual activity of the WT (serum + IgG / serum—IgG) and "b" is the percentage residual activity of the mutant (serum + IgG / serum -IgG).

FIGS. **15-18** show for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant **2316** for sera TD and SL, mutant **2294** for serum GC, mutant **403** for serum FS and mutant **2275** for serum PR.

Patients' sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create

Column 10, Line 42 through Column 14, Line 47 cont'd.

combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIIIs produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least **50**% of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). Figure **19** shows comparative data of raw and specific activities of **30** mutants selected in the secondary screen.

The eight FVIII Alanine mutants **2175**, **2199**, **2200**, **2215**, **2251**, **2252**, **2278** and **2316** displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. 3 depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants **2177**, **2183**, **2186**, **2191**, **2196**, **2204**, **2205**, **2206**, **2213**, **2217**, **2235**, **2258**, **2264**, **2268** and **2269** displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15mutants are given in FIG. **4**. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

Example 5: Selection and combination of the best single mutants selected in the secondary screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophiliac patients with inhibitors;
- raw coagulant activity at least **100**% relative to non-mutated FVIII; and
- reproducibly good level of expression.

The eight selected mutants were mutants **409**, **462**, **507** and **629** in the A**2** domain and mutants **2289**, **2294**, **2312** and **2316** in the C**2** domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in FIG. **19**. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations **409**, **462**, **507**, **629**, **2289**, **2294**, **2312** and **2316** (six A**2** double mutants +six C**2** double mutants +sixteen A**2**-C**2**
Column 10, Line 42 through Column 14, Line 47 cont'd.

double mutants presented in FIG. **20**) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-**7** mammalian cells as described in Example 2. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophiliac patients. The A**2** double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C**2** domain mutations (six C**2** double mutants +sixteen A**2**-C**2** double mutants) displayed an insignificant or null abolition to inhibition.

FIG. **21** shows the specific activities of the six A**2** double mutants and their percentage of abolition to inhibition by sera from four hemophiliac patients TD, GC, SL and PR calculated as in Example **4**. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A**2** domain. The choice was therefore based on the combination of the four mutations **409**, **507**, **462** and **629**. Triple mutants and the quadruple mutant comprising these four mutations **409**, **507**, **462** and **629** were also constructed.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

Table 1: Primary screen results; list of 158 Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

Table 2: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. **5**.

Table 3: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

Table 4: List of all FVIII double mutants produced from the eight single mutants FVIII409A, FVIII462A, FVIII507A, FVIII629A, FVIII2289A, FVIII2294A, FVIII2312A and FVIII2316A.

Table 5: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

DESCRIPTION OF THE INVENTION"

should read

--**FIG. 21:** Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

DESCRIPTION OF THE INVENTION--.

<u>Column 27,</u>
Line 44, "responsible of its" should read --responsible for its--.
<u>Column 28,</u>

Line 20, “from a patient” should read --from one patient--.

Column 31,
Line 48, “of a continuously use” should read --of a continuous use--.
Lines 55-56, “might be encompass for a” should read --might encompass a--.

Column 32,
Line 61, “propose to use of the” should read --propose to use the--.
Lines 66-67, “decrease for the control” should read --decrease the control--.

Column 33,
Line 55, “consisted in” should read --consisted of--.

Column 35,
Line 33, “Table 1 shows” should read --Figures 12-14 show--.

Column 36,
Line 19, “Table 2 shows” should read --Figures 15-18 show--.
Line 52, “Table 3 shows” should read --Figure 19 shows--.

Column 37,
Line 41, “shown in Table 3.” should read --shown in Figure 19.--.
Line 46, “in Table 4)” should read --in Figure 20)--.
Line 60, “Table 5 shows” should read --Figure 21 shows--.

Column 39,
Line 35, “about 27±1” should read --about 27 ± 11--.
Line 65, “added (Iris” should read --added (Tris--.